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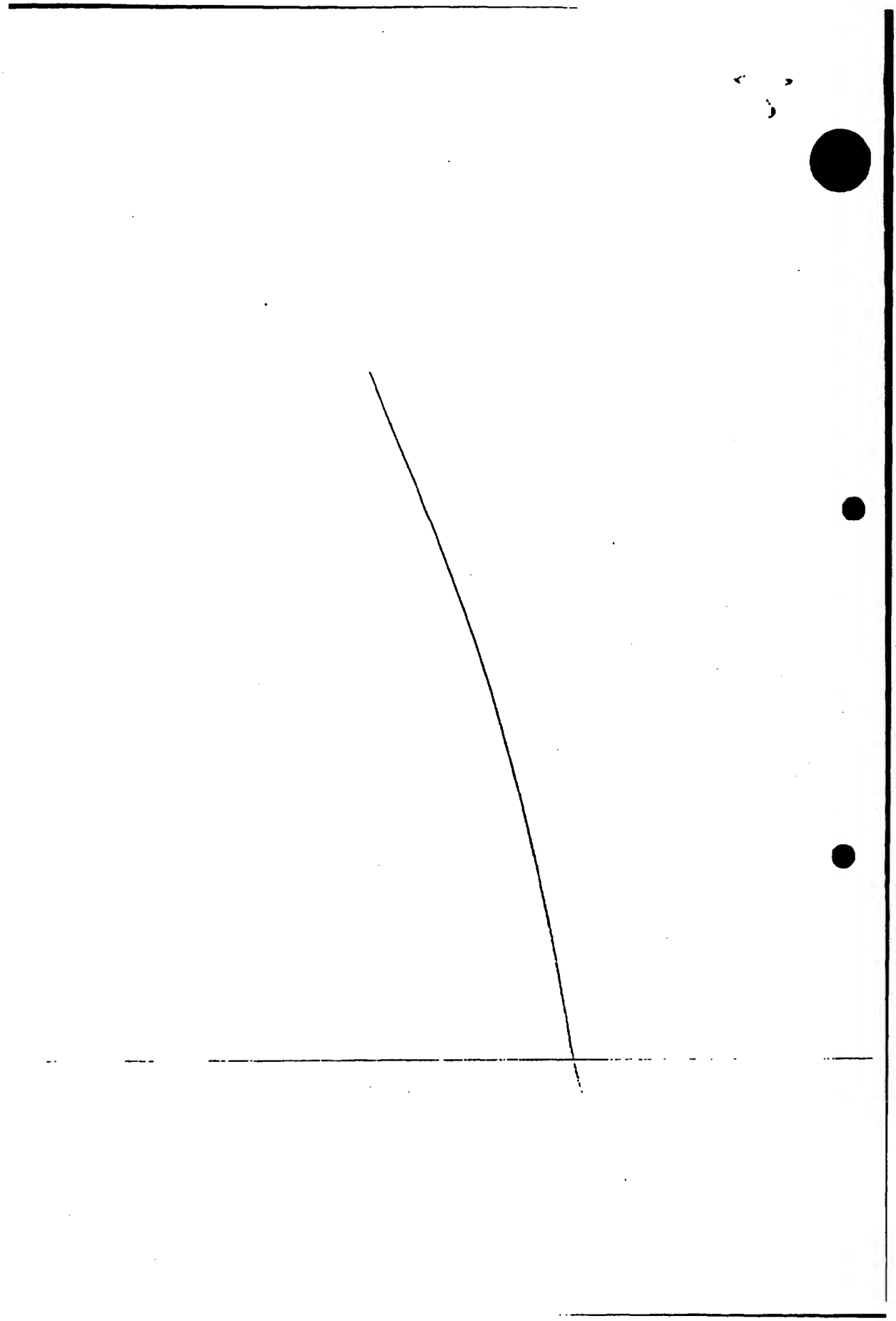

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PROTEINS

BACKGROUND OF THE PRESENT INVENTION

The present invention relates to proteins.

In particular, the present invention relates to the isolation and characterisation of an endogenous endo-β-1,4-xylanase inhibitor in wheat flour and its effect on different xylanases. The present invention also relates to xylanases identified by a screen using the inhibitor and to novel xylanases identified thereby.

BACKGROUND ART

Xylanases have been used in bakery for several years.

In this regard, it is known that wheat flour contains arabinoxylan originating from the endosperm cell walls. The amount of arabinoxylan in the flour differs depending on the origin of the flour - for example, see Rouau *et al*, Journal of Cereal Science (1994), 19, 259-272 *Effect of an Enzyme Preparation Containing Pentosanases on the Bread-making Quality of Flour in Relation to Changes in Pentosan Properties*; Fincher and Stone. (1986) *Advances in Cereal Technology*, Vol. VIII (Why Pomeranz, Ed.) AACC, St Paul, Minnesota, 207-295; and Meuser and Suckow (1986), *Chemistry and Physics of Baking* (J.M.V. Blanchard, P J Frasier and T Gillard, Eds.) Royal Society of Chemistry, London, 42-61. Typically the amount of arabinoxylan can vary from 2-5% ((w/w) based on flour dry weight).

Fincher and Stone (1986) report 70% of the polysaccharides in the endo sperm cell wall are arabinoxylan. A characteristic feature of arabinoxylan is its ability to bind to water. Part of the arabinoxylan is water insoluble pentosan (WIP) and part is water soluble pentosan (WSP). Experimental results have shown a correlation between degradation of WIP to high molecular weight (HMW) water soluble polymers and bread volume.

During the production of a bakery product, it is known that using a xylanase at a proper dosage may result in a more stable dough system (which will typically comprise salt, flour and water) and a better volume of yeast in, for example, raised bread.

In this respect, a good xylanase for increasing bread volume should solubilise WIP giving an increased viscosity in the dough liquid without further degradation of WSP into xylose oligomers. This degradation of WIP into low molecular weight (LMW) WSP is believed to be detrimental for the dough properties and may give rise to stickiness (Rouau *et al* and McCleary (1986) *International Journal of Biological Macro Molecules, 8, 349-354*).

US-A-5306633 discloses a xylanase obtained from a *Bacillus subtilis* strain. Apparently, this xylanase may improve the consistency and increase the volume of bread and baked goods containing the same.

Another xylanase from *Bacillus subtilis* has been isolated and sequenced (see Paice, M.G., Bourbonnais, R., Desrochers, M., Jurasek, L. and Yaguchi, M. *A xylanase gene from Bacillus subtilis: nucleotide sequence and comparison with B. pumilus gene,* Arch. Microbiol. 144, 201-206 (1986)).

It has been considered for some time now that bacterial xylanases would produce very sticky dough. Hence, one would normally expect the xylanases of *Bacillus subtilis* - such as that of US-A-5306633 - to produce a very sticky dough.

Prior art enzymes which caused stickiness had to be used in carefully controlled amounts such that stickiness would not adversely affect handling to a degree that effective commercial handling was hampered. However, the need to carefully control dosage prohibited the addition of xylanase directly to flour prior to production of the dough. It was therefore necessary with prior art systems to add the xylanase in a very controlled manner during the production of the dough.

To date, fungal xylanases have been typically used in baking. For example, J Maat *et al.*, Xylans and Xylanases, edited by J Visser *et al*, 349-360, *Xylanases and their application in bakery*, teach a β-1,4-xylanase produced by an *Aspergillus Niger* var. awarmori strain. According to these authors, the fungal xylanase is effective in increasing the specific volume of breads, without giving rise to a negative side effect on dough handling (stickiness of the dough) as can be observed with xylanases derived from other fungal or from bacterial sources.

It has been proposed by W Debyser *et al.*, (J. Am. Soc. Brew. Chem. 55(4), 153-156, 1997, *Arabinoxylan Solubilization and Inhibition of the Barely Malt Xylanolytic System by Wheat During Mashing with Wheat Wholemeal Adjunt: Evidence for a New Class of Enzyme Inhibitors in Wheat)*, that xylanase inhibitors may be present in wheat. The inhibitor discussed by W Debyser *et al.* was not isolated. Furthermore, it is not disclosed by W Debyser *et al.* whether the inhibitor is endogenous or microbiological. Moreover, no chemical data were presented for this inhibitor.

The presence of xylanase inhibitor in wheat flour has also recently been discussed by X Rouau and A Surget, (Journal of Cereal Science, 28 (1998) 63-70, *Evidence for the Presence of a Pentosanase Inhibitor in Wheat Flours)*. Similar to Debyser *et al.*, Rouau and Surget believed that they have identified the existence of a thermolabile compound in the soluble fraction of wheat flours, which limited the action of an added pentosanase. Also similarly to Debyser *et al.*, these authors did not isolate an inhibitor and were unable to conclude whether the inhibitor is endogenous or is of microbial origin. Likewise, no chemical data were presented for this inhibitor.

Thus, a known problem in the art is how to prepare baked goods from a dough which does not have adverse handling properties. A more particular problem is how to provide a dough ~~which is non-sticky - i.e. a dough that is not so sticky that it causes handling~~ and processing problems.

The present invention seeks to provide a solution to these problems.

SUMMARY ASPECTS OF THE PRESENT INVENTION

Aspects of the present invention are presented in the claims and in the following commentary.

In brief, some aspects of the present invention relate to:

1. An endogenous endo-β-1,4-xylanase inhibitor - including nucleotide sequences coding therefor and the amino acid sequences thereof, as well as variants, homologues, or fragments thereof.

2. Assay methods for determining the effect of the β-1,4-xylanase inhibitor on different xylanases.

3. Assay methods for determining the effect of different xylanases in dough.

4. Assay methods for determining the effect of glucanase(s) on different xylanases.

5. Novel xylanases - including nucleotide sequences coding therefor and the amino acid sequences thereof, as well as variants, homologues, or fragments thereof.

6. Novel uses of xylanases.

7. Foodstuffs prepared with xylanases.

Other aspects concerning the amino acid sequence of the present invention and/or the nucleotide sequence of the present invention include: a construct comprising or capable of expressing the sequences of the present invention; a vector comprising or capable of expressing the sequences of the present invention; a plasmid comprising or capable of expressing the sequences of present invention; a tissue comprising or capable of expressing the sequences of the present invention; an organ comprising or capable of expressing the sequences of the present invention; a transformed host comprising or capable of expressing

the sequences of the present invention; a transformed organism comprising or capable of expressing the sequences of the present invention. The present invention also encompasses methods of expressing the same, such as expression in a micro-organism; including methods for transferring same.

Aspects of the present invention are now discussed under appropriate section headings. For the sake of convenience, generally applicable teachings for the aspects of the present invention may be found in the sections titled "General Definitions" and "General Teachings".

GENERAL DEFINITIONS

The term "wheat flour" as used herein is a synonym for the finely-ground meal of wheat or other grain. Preferably, however, the term means flour obtained from wheat *per se* and not from another grain. Thus, and unless otherwise expressed, references to "wheat flour" as used herein preferably mean references to wheat flour *per se* as well as to wheat flour when present in a medium, such as a dough.

The term "xylanase" is used in its normal sense - e.g. an enzyme that is *inter alia* capable of catalysing the solubilisation of WIP which may be present in wheat.

An assay for determining endo-β-1,4-xylanase activity is presented later. For convenience, this assay is called the "Xylanase Assay".

The term "nucleotide sequence" in relation to the present invention includes genomic DNA, cDNA, recombinant DNA (i.e. DNA prepared by use of recombinant DNA techniques), synthetic DNA, and RNA - as well as combinations thereof. Preferably it means DNA. ~~The nucleotide sequences of the present invention may be single or double stranded.~~

The nucleotide sequences of the present invention may include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides

are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the nucleotide sequences described herein may be modified by any method available in the art. Such modifications may be carried out in to enhance the *in vivo* activity or life span of nucleotide sequences of the present invention.

The terms "variant" or "homologue" with respect to the nucleotide sequence of the present invention and the amino acid sequence of the present invention are synonymous with allelic variations of the sequences.

In particular, the term "homology" as used herein may be equated with the term "identity". Here, sequence homology can be determined by a simple "eyeball" comparison (i.e. a strict comparison) of any one or more of the sequences with another sequence to see if that other sequence has at least 75% identity to the sequence(s). Relative sequence homology (i.e. sequence identity) can also be determined by commercially available computer programs that can calculate % homology between two or more sequences. A typical example of such a computer program is CLUSTAL.

Sequence homology (or identity) may even be determined using any suitable homology algorithm, using for example default parameters. Advantageously, the BLAST algorithm is employed, with parameters set to default values. The BLAST algorithm is described in detail at http://www.ncbi.nih.gov/BLAST/blast_help.html, which is incorporated herein by reference. The search parameters are defined as follows, and are advantageously set to the defined default parameters.

Advantageously, "substantial homology" when assessed by BLAST equates to sequences ~~which match with an EXPECT value of at least about 7, preferably at least about 9~~ and most preferably 10 or more. The default threshold for EXPECT in BLAST searching is usually 10.

BLAST (Basic Local Alignment Search Tool) is the heuristic search algorithm employed by the programs blastp, blastn, blastx, tblastn, and tblastx; these programs ascribe significance to their findings using the statistical methods of Karlin and Altschul (see http://www.ncbi.nih.gov/BLAST/blast_help.html) with a few enhancements. The BLAST programs were tailored for sequence similarity searching, for example to identify homologues to a query sequence. The programs are not generally useful for motif-style searching. For a discussion of basic issues in similarity searching of sequence databases, see Altschul et al (1994) Nature Genetics 6:119-129.

The five BLAST programs available at http://www.ncbi.nlm.nih.gov perform the following tasks:

blastp compares an amino acid query sequence against a protein sequence database;

blastn compares a nucleotide query sequence against a nucleotide sequence database;

blastx compares the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database;

tblastn compares a protein query sequence against a nucleotide sequence database dynamically translated in all six reading frames (both strands).

tblastx compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database.

BLAST uses the following search parameters:

~~HISTOGRAM Display a histogram of scores for each search; default is yes. (See~~ parameter H in the BLAST Manual).

DESCRIPTIONS Restricts the number of short descriptions of matching sequences reported to the number specified; default limit is 100 descriptions. (See parameter V in the manual page). See also EXPECT and CUTOFF.

ALIGNMENTS Restricts database sequences to the number specified for which high-scoring segment pairs (HSPs) are reported; the default limit is 50. If more database sequences than this happen to satisfy the statistical significance threshold for reporting (see EXPECT and CUTOFF below), only the matches ascribed the greatest statistical significance are reported. (See parameter B in the BLAST Manual).

EXPECT The statistical significance threshold for reporting matches against database sequences; the default value is 10, such that 10 matches are expected to be found merely by chance, according to the stochastic model of Karlin and Altschul (1990). If the statistical significance ascribed to a match is greater than the EXPECT threshold, the match will not be reported. Lower EXPECT thresholds are more stringent, leading to fewer chance matches being reported. Fractional values are acceptable. (See parameter E in the BLAST Manual).

CUTOFF Cutoff score for reporting high-scoring segment pairs. The default value is calculated from the EXPECT value (see above). HSPs are reported for a database sequence only if the statistical significance ascribed to them is at least as high as would be ascribed to a lone HSP having a score equal to the CUTOFF value. Higher CUTOFF values are more stringent, leading to fewer chance matches being reported. (See parameter S in the BLAST Manual). Typically, significance thresholds can be more intuitively managed using EXPECT.

MATRIX Specify an alternate scoring matrix for BLASTP, BLASTX, TBLASTN and ~~TBLASTX. The default matrix is BLOSUM62 (Henikoff & Henikoff, 1992). The~~ valid alternative choices include: PAM40, PAM120, PAM250 and IDENTITY. No alternate scoring matrices are available for BLASTN; specifying the MATRIX directive in BLASTN requests returns an error response.

STRAND Restrict a TBLASTN search to just the top or bottom strand of the database sequences; or restrict a BLASTN, BLASTX or TBLASTX search to just reading frames on the top or bottom strand of the query sequence.

FILTER Mask off segments of the query sequence that have low compositional complexity, as determined by the SEG program of Wootton & Federhen (1993) Computers and Chemistry 17:149-163, or segments consisting of short-periodicity internal repeats, as determined by the XNU program of Claverie & States (1993) Computers and Chemistry 17:191-201, or, for BLASTN, by the DUST program of Tatusov and Lipman (see http://www.ncbi.nlm.nih.gov). Filtering can eliminate statistically significant but biologically uninteresting reports from the blast output (e.g., hits against common acidic-, basic- or proline-rich regions), leaving the more biologically interesting regions of the query sequence available for specific matching against database sequences.

Low complexity sequence found by a filter program is substituted using the letter "N" in nucleotide sequence (e.g., "NNNNNNNNNNNNN") and the letter "X" in protein sequences (e.g., "XXXXXXXXX").

Filtering is only applied to the query sequence (or its translation products), not to database sequences. Default filtering is DUST for BLASTN, SEG for other programs.

It is not unusual for nothing at all to be masked by SEG, XNU, or both, when applied to sequences in SWISS-PROT, so filtering should not be expected to always yield an effect. Furthermore, in some cases, sequences are masked in their entirety, indicating that the statistical significance of any matches reported against the unfiltered query sequence should be suspect.

NCBI-gi Causes NCBI gi identifiers to be shown in the output, in addition to the accession and/or locus name.

Most preferably, sequence comparisons are conducted using the simple BLAST search algorithm provided at http://www.ncbi.nlm.nih.gov/BLAST.

Other computer program methods to determine identify and similarity between the two sequences include but are not limited to the GCG program package (Devereux et al 1984 Nucleic Acids Research 12: 387 and FASTA (Atschul et al 1990 J Molec Biol 403-410).

The present invention also encompasses nucleotide sequences that are complementary to the sequences presented herein, or any derivative, fragment or derivative thereof. If the sequence is complementary to a fragment thereof then that sequence can be used a probe to identify similar coding sequences in other organisms etc.

The present invention also encompasses nucleotide sequences that are capable of hybridising to the sequences presented herein, or any derivative, fragment or derivative thereof.

The present invention also encompasses nucleotide sequences that are capable of hybridising to the sequences that are complementary to the sequences presented herein, or any derivative, fragment or derivative thereof.

The term "complementary" also covers nucleotide sequences that can hybridise to the nucleotide sequences of the coding sequence.

The term "variant" also encompasses sequences that are complementary to sequences that are capable of hydridising to the nucleotide sequences presented herein.

Preferably, the term "variant" encompasses sequences that are complementary to sequences that are capable of hydridising under stringent conditions (e.g. 65°C and 0.1xSSC {1xSSC = 0.15 M NaCl, 0.015 Na_3 citrate pH 7.0}) to the nucleotide sequences presented herein.

The present invention also relates to nucleotide sequences that can hybridise to the nucleotide sequences of the present invention (including complementary sequences of those presented herein).

The present invention also relates to nucleotide sequences that are complementary to sequences that can hybridise to the nucleotide sequences of the present invention (including complementary sequences of those presented herein).

The term "hybridization" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" (Coombs J (1994) Dictionary of Biotechnology, Stockton Press, New York NY) as well as the process of amplification as carried out in polymerase chain reaction technologies as described in Dieffenbach CW and GS Dveksler (1995, PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview NY).

Also included within the scope of the present invention are polynucleotide sequences that are capable of hybridizing to the nucleotide sequences presented herein under conditions of intermediate to maximal stringency. Hybridization conditions are based on the melting temperature (Tm) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.

Maximum stringency typically occurs at about Tm-5°C (5°C below the Tm of the probe); high stringency at about 5°C to 10°C below Tm; intermediate stringency at about 10°C to 20°C below Tm; and low stringency at about 20°C to 25°C below Tm. As will be understood by those of skill in the art, a maximum stringency hybridization can be used to identify or detect identical polynucleotide sequences while an intermediate (or low) stringency hybridization can be used to identify or detect similar or related polynucleotide sequences.

In a preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequence of the present invention under stringent conditions (e.g. 65°C and 0.1xSSC).

ENDOGENOUS ENDO-β-1,4-XYLANASE INHIBITOR

In one aspect the present invention provides an endogenous endo-β-1,4-xylanase inhibitor that is obtainable from wheat flour.

In our studies, we have found that the inhibitor has a MWT of about 34 kDa (as measured by gel filtration or SDS page) and that it has a pI of about 6.

In one aspect of the present invention, the inhibitor is in an isolated form and/or in a substantially pure form. Here, the term "isolated" means that the inhibitor is not in its natural environment.

Sequence analysis to date has revealed the that the inhibitor has at least one or both of the sequences presented as SEQ. ID No. 1 and SEQ. ID No. 2.

Thus, the present invention encompasses an endo-β-1,4-xylanase inhibitor which comprises the amino acid sequence presented as SEQ. ID No. 1 or a variant, homologue, or fragment thereof and/or the amino acid sequence presented as SEQ. ID No. 2 or a variant, homologue, or fragment thereof.

The terms "variant", "homologue" or "fragment" in relation to the inhibitor of the present invention include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acid from or to the sequence providing the resultant amino acid sequence has xylanase inhibitory action, preferably having at least the same activity as an inhibitor comprising the amino acid sequence shown as SEQ. I.D. No. 1 and/or SEQ ID No. 2. In particular, the term "homologue" covers homology with respect to structure and/or function providing the resultant inhibitor has xylanase inhibitory action, preferably having at least the same activity of an inhibitor comprising sequence

shown as sequence shown as SEQ. I.D. No. 1 and/or SEQ ID No. 2. With respect to sequence homology (i.e. sequence similarity or sequence identity), preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to the sequence shown in the attached sequence listings. More preferably there is at least 95%, more preferably at least 98%, homology to the sequence shown in the attached sequence listings.

This aspect of the present invention is advantageous for a number of reasons.

By way of example, by now knowing the chemical identity of an endogenous endo-β-1,4-xylanase inhibitor workers can now determine the quantity of the inhibitor in, for example, a wheat flour. This would then enable workers to select one or more appropriate xylanases for addition to the wheat flour and/or select appropriate amounts of one or more xylanases for addition to the wheat flour.

Thus, the present invention provides a method comprising: (a) determining the amount of inhibitor in a wheat flour; (b) selecting a suitable xylanase for addition to the wheat flour and/or selecting a suitable amount of a xylanase for addition to the wheat flour; and (c) adding the suitable xylanase and/or suitable amount of the xylanase to the wheat flour. For convenience, we shall call this method the "Inhibitor Amount Determination Method".

Detection of the amount of inhibitor can be determined by standard chemical techniques, such as by analysis of solid state NMR spectra. The amount of inhibitor may even be determined by use of xylanase enzymes that are known to be detrimentally affected by the inhibitor. In this last aspect, it would be possible to take a sample of the wheat flour and add it to a known quantity of such a xylanase. At a certain time point the activity of the xylanase can be determined, which resultant activity can then be correlated to an amount of inhibitor in the wheat flour.

Thus, the present invention also encompasses the use of the combination of a xylanase and the inhibitor as a means to calibrating and/or determining the quantity of inhibitor in a wheat flour sample.

Antibodies to the inhibitor can be used to screen wheat flour samples for the presence of the inhibitor of the present invention. The antibodies may even be used to isolate amounts of the inhibitor from a wheat flour sample.

ASSAY METHODS FOR DETERMINING THE EFFECT OF THE β-1,4-XYLANASE INHIBITOR ON DIFFERENT XYLANASES

There is an additional important use of the inhibitor of the present invention. In this respect, the inhibitor can be used in an assay/screen to identify xylanases that are resistant to the inhibitor.

A suitable Protocol for determining the degree of inhibition by the inhibitor is presented later on. For convenience, we shall call this Protocol "Inhibitor Assay Protocol".

Thus, the present invention provides a method for determining the degree of resistance of a xylanase to a xylanase inhibitor, wherein the method comprises: (a) contacting a xylanase of interest with the inhibitor; and (b) determining whether the inhibitor inhibits the activity of the xylanase of interest. For convenience, we shall call this method the "Inhibitor Assay Method".

Here, the term "resistant" means that the activity of the xylanase is not totally inhibited by the inhibitor. In other words, the inhibitor can be used in an assay/screen to identify xylanases that are not detrimentally affected by the inhibitor.

Thus, the term "degree of resistance" in relation to the xylanase *vis-a-vis* the xylanase inhibitor is synonymous with the degree of non-inhibition of the activity of a xylanase by ~~the xylanase inhibitor. Thus, a xylanase that has a high degree of resistance~~ to the xylanase inhibitor is akin to a high degree of non-inhibition of a xylanase by the xylanase inhibitor.

The present invention also encompasses a process comprising the steps of (a) performing the Inhibitor Assay Method; (b) identifying one or more xylanases having a high degree of resistance to the inhibitor; (c) preparing a quantity of those one or more identified xylanases.

Suitable identified xylanases can then be used to prepare a foodstuff, in particular a dough to make a bakery product.

In addition, by identifying a xylanase that is resistant to some extent to the inhibitor (i.e. a xylanase that is not inhibited as much as other xylanases), it is possible to add less of that identified xylanase to a medium for subsequent utilisation thereof. End uses for the xylanases can include any one or more of the preparation of foodstuffs, proetin and starch production, paper production and pulp processing etc.

Thus, the present invention also encompasses a process comprising the steps of: (a) performing the Inhibitor Assay Method; (b) identifying one or more xylanases having a high degree of resistance to the inhibitor; and (c) preparing a dough comprising the one or more identified xylanases.

In the course of the experiments relating to the present invention, we surprisingly found that bacterial xylanases were able to be resistant to the inhibitor, in the sense that their activity was not compeletly abolished. In some cases, the xylanases exhibited very favourable resistance to the inhibitor.

ASSAY METHODS FOR DETERMINING THE EFFECT OF DIFFERENT XYLANASES IN DOUGHS

When some bacterial xylanases that had been identified as being suitable by the Inhibitor Assay Method were present in a dough mixture, we surprisingly found that the dough mixture was not as sticky as a dough mixture comprising a fungal xylanase. These results were completely unexpected in view of the teachings of the prior art.

Thus, the present invention provides a further assay method for identifying a bacterial xylanase or mutant thereof suitable for use in the preparation of a baked foodstuff. The method comprises (a) incorporating a bacterial xylanase of interest in a dough mixture; and (b) determining the stickiness of the resultant dough mixture; such that the bacterial xylanase or mutant thereof is suitable for use in the preparation of a baked foodstuff if the resultant dough mixture has a stickiness that is less than a similar dough mixture comprising a fungal xylanase. For convenience, we shall call this method the "Stickiness Assay Method".

Thus, the present invention also provides a process comprising the steps of: (a) performing the Stickiness Assay Method; (b) identifying one or more xylanases suitable for use in the preparation of a baked foodstuff; (c) preparing a quantity of those one or more identified xylanases.

A suitable Protocol for determining the stickiness of a dough is presented later on. For convenience, we shall call this Protocol the "Stickiness Protocol". In accordance with the present invention a dough comprising a xylanase according to the present invention that is less sticky than a dough comprising a fungal xylanase may be called, on occassion, a "non-sticky dough".

If a bacterial xylanase shows favourable properties - in that it does not produce a dough that is as sticky as a dough comprising a fungal xylanase - then that xylanase may be used to prepare a foodstuff, such as a dough for preparing a bakery product.

Thus, the present invention also provides a process comprising the steps of: (a) performing the Stickiness Assay Method; (b) identifying one or more xylanases suitable for use in the preparation of a baked foodstuff; and (c) preparing a dough comprising the ~~one or more identified xylanases.~~

ASSAY METHODS FOR DETERMINING THE EFFECT OF GLUCANASE(S) ON DIFFERENT XYLANASES

In the course of the experiments relating to the present invention, we also found that the presence of glucanase enzymes in certain amounts could have a detrimental effect on the xylanases.

Thus, in one aspect, it is advantageous not to have detrimental levels of glucanase enzymes in the xylanase preparation - such as the medium used to prepare or extract the xylanase enzymes. In addition, for some aspects, it is advantageous not to have detrimental levels of glucanase enzymes in a medium that is to be used to prepare a foodstuff which medium will contain the xylanase. Here, the term "detrimental level" means an amount of glucanase is present such that the benefits from the xylanase are masked by the adverse effect of the glucanase enzymes.

Thus, the present invention provides a further assay method for identifying a xylanase composition (such as a xylanase preparation) or a medium in which a xylanase is to be prepared or a medium to which a xylanase is to be added that is to be suitable for use in the preparation of a baked foodstuff, the method comprising (a) providing a composition containing the xylanase of interest or a medium in which the xylanase is to be prepared or a medium to which the xylanase is to be added; and (b) determining the presence of active glucanase enzyme(s) in the composition or medium; such that if there is at most a low level of active glucanase enzyme(s) in the composition or medium then that composition or medium is suitable for the preparation of a baked foodstuff. For convenience, we shall call this method the "Glucanase Assay Method".

The present invention also provides a process comprising the steps of: (a) performing the Glucanase Assay Method; (b) identifying one or more compositions or mediums suitable for use in the preparation of a baked foodstuff; (c) preparing a quantity of those one or more identified compositions or mediums.

A suitable Protocol for determining the effect of glucanases is presented later on. For convenience, we shall call this Protocol the "Glucanase Protocol".

If the composition or medium shows favourable properties - in the sense that the beneficial effects associated with the xylanase are not completely masked by the presence of detrimental amounts of glucanase enzymes - then that composition or medium may be used to prepare a foodstuff, preferably dough that is used to make a bakery product.

Thus, the present invention also encompasses a process comprising the steps of: (a) performing the Glucanase Assay Method; (b) identifying one or more identified compositions or mediums suitable for use in the preparation of a baked foodstuff; and (c) preparing a dough comprising the one or more identified identified compositions or mediums.

Thus, the present invention covers a xylanase preparation, wherein the xylanase preparation is substantially free of glucanase enzyme(s).

COMBINATION ASSAYS

The present invention also encompasses suitable combinations of the assays of the present invention.

In this respect, the present invention includes a combination method comprising two or more of the following steps: a first step comprising the Inhibitor Amount Determination Method, a second step comprising the Inhibitor Assay Method, a third step comprising the Stickiness Assay Method; and a fourth step comprising the Glucanase Assay Method. In the combination method, the steps can occur in any order and need not necessarily occur simulataneously or consecutively.

NOVEL XYLANASES

As indicated above, the present invention provides a suitable assay for identifying xylanases that can be used in the preparation of foodstuffs, in particular doughs for use in the preparation of bakery products.

In this respect, we have identified three new xylanases that are suitable for the preparation of foodstuffs, in particular doughs for use in the preparation of bakery products.

Thus, the present invention also includes an amino acid sequence comprising any one of the amino acid sequences presented as SEQ ID No. 7, SEQ ID No. 9 or SEQ ID No. 11, or a variant, homologue or fragment thereof.

The terms "variant", "homologue" or "fragment" in relation to the xylanase of the present invention include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acid from or to the sequence providing the resultant amino acid sequence has xylanase activity, preferably having at least the same activity comprising any one of the amino acid sequences presented as SEQ ID No. 7, SEQ ID No. 9 or SEQ ID No. 11. In particular, the term "homologue" covers homology with respect to structure and/or function providing the resultant protein has xylanase activity, preferably at least the same activity of any one of the amino acid sequences presented as SEQ ID No. 7, SEQ ID No. 9 or SEQ ID No. 11. With respect to sequence homology (i.e. sequence similarity or sequence identity), preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to the sequence shown in the attached sequence listings. More preferably there is at least 95%, more preferably at least 98%, homology to the sequence shown in the attached sequence listings.

Preferably, the xylanase comprises the sequence presented as SEQ ID No. 7 or SEQ ID No. 11, or a variant, homologue or fragment thereof.

The present invention also encompasses a nucleotide sequence encoding the amino acid sequence of the present invention.

Preferably, the nucleotide sequence of the present invention is selected from:

(a) a nucleotide sequence comprising any one of the nucleotide sequences presented as SEQ ID No. 8, SEQ ID No. 10 or SEQ ID No. 12, or a variant, homologue or fragment thereof;

(b) any one of the nucleotide sequences presented as SEQ ID No. 8, SEQ ID No. 10 or SEQ ID No. 12, or the complement thereof;

(c) a nucleotide sequence capable of hybridising any one of the nucleotide sequences presented as SEQ ID No. 8, SEQ ID No. 10 or SEQ ID No. 12, or a fragment thereof;

(d) a nucleotide sequence capable of hybridising to the complement any one of the nucleotide sequences presented as SEQ ID No. 8, SEQ ID No. 10 or SEQ ID No. 12, or a fragment thereof; and

(e) a nucleotide sequence which is degenerate as a result of the genetic code to the nucleotides defined in (a), (b), (c) or (d).

The terms "variant", "homologue" or "fragment" in relation to the nucleotide sequence of the present invention include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence providing the resultant nucleotide sequence codes for an amino acid sequence has xylanase activity, preferably having at least the same activity comprising any one of the amino acid sequences presented as SEQ ID No. 7, SEQ ID No. 9 or SEQ ID No. 11. In particular, the term "homologue" covers homology with respect to structure and/or function providing the resultant expressed protein has xylanase activity, preferably at least the same activity of any one of the amino acid sequences presented as SEQ ID No. 7, SEQ ID No. 9 or SEQ ID No. 11. With respect to sequence homology (i.e. sequence similarity or sequence identity), preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to the sequence shown as SEQ ID No. 8, SEQ ID No. 10 or SEQ ID No. 12 in the attached sequence listings. More preferably there is at least 95%, more preferably at least 98%, homology to the sequence shown in the attached sequence listings.

Preferably, the nucleotide sequence of the present invention comprises the sequence presented as SEQ ID No. 8 or SEQ ID No. 12, or a variant, homologue or fragment thereof.

NOVEL USES OF XYLANASES

As indicated above, the present invention also provides a suitable assay for identifying xylanases that can be used in the preparation of non-sticky doughs (as defined herein) for use in the preparation of bakery products.

In this respect, we have identified certain xylanases, both known and new bacterial xylanases, that are suitable for the preparation of foodstuffs, in particular doughs for use in the preparation of bakery products.

Thus, the present invention covers a non-sticky dough (as herein defined) which dough comprises a xylanase identifiable by the assay of the present invention. Preferably, the xylanase has an amino acid sequence presented as any one of SEQ ID No.s 3, 5, 7, 9, 11, or a variant, derivative or homologue thereof. More preferably, the xylanase has an amino acid sequence presented as any one of SEQ ID No.s 5, 7, 9, 11, or a variant, derivative or homologue thereof.

In contrast to the prior art systems, the present invention provides for the possibility of the addition of xylanase directly to flour prior to production of the dough. Thus, a single batch a flour/xylanase mixture may be delivered to the dough producer. Moreover, the dough producer does not require dosing equipment to be able to obtain a readily handable dough.

FOODSTUFFS PREPARED WITH XYLANASES

The present invention provides a means of identifying suitable xylanases for use in the manufacture of a foodstuff. Typical foodstuffs, which also include animal feed, include dairy products, meat products, poultry products, fish products and bakery products.

Preferably, the foodstuff is a bakery product. Typical bakery (baked) products incorporated within the scope of the present invention include bread - such as loaves, rolls, buns, pizza bases etc. - pretzels, tortillas, cakes, cookies, biscuits, krackers etc.

GENERAL TEACHINGS

In the following commentary references to "nucleotide sequence of the present invention" and "amino acid sequence of the present invention" refer respectively to any one or more of the nucleotide sequences presented herein and to any one or more of the amino acid sequences present herein.

Amino Acid Sequence/Polypeptide Sequence

The term "amino acid sequence of the present invention" is synonymous with the phrase "polypeptide sequence of the present invention".

Polypeptides of the present invention also include fragments of the presented amino acid sequence and variants thereof. Suitable fragments will be at least 5, e.g. at least 10, 12, 15 or 20 amino acids in size.

Polypeptides of the present invention may also be modified to contain one or more (e.g. at least 2, 3, 5, or 10) substitutions, deletions or insertions, including conserved substitutions.

Conserved substitutions may be made according to the following table which indicates conservative substitutions, where amino acids on the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y
OTHER		N Q D E

Polypeptides of the present invention may be in a substantially isolated form. It will be understood that the polypeptide may be mixed with carriers or diluents which will not interfere with the intended purpose of the polypeptide and still be regarded as substantially isolated. A polypeptide of the present invention may also be in a substantially purified form, in which case it will generally comprise the polypeptide in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the polypeptide in the preparation is a polypeptide of the present invention. Polypeptides of the present invention may be modified for example by the addition of histidine residues to assist their purification or by the addition of a signal sequence to promote their secretion from a cell as discussed below.

Polypeptides of the present invention may be produced by synthetic means (e.g. as described by Geysen *et al.*, 1996) or recombinantly, as described below.

The use of suitable host cells - such as yeast, fungal and plant host cells - may provide for such post-translational modifications (e.g. myristolation, glycosylation, truncation, lapidation and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the present invention.

Nucleotide Sequence/Polynucleotide Sequence

The term "nucleotide sequence of the present invention" is synonymous with the phrase "polynucleotide sequence of the present invention".

Polynucleotides of the present invention include nucleotide acid sequences encoding the polypeptides of the present invention. It will appreciated that a range of different polynucleotides encode a given amino acid sequence as a consequence of the degeneracy of the genetic code.

By knowledge of the amino acid sequences set out herein it is possible to devise partial and full-length nucleic acid sequences such as cDNA and/or genomic clones that encode the polypeptides of the present invention. For example, polynucleotides of the present invention may be obtained using degenerate PCR which will use primers designed to target sequences encoding the amino acid sequences presented herein. The primers will typically contain multiple degenerate positions. However, to minimise degeneracy, sequences will be chosen that encode regions of the amino acid sequences presented herein containing amino acids such as methionine which are coded for by only one triplet. In addition, sequences will be chosen to take into account codon usage in the organism whose nucleic acid is used as the template DNA for the PCR procedure. PCR will be used at stringency conditions lower than those used for cloning sequences with single sequence (non-denegerate) primers against known sequences.

Nucleic acid sequences obtained by PCR that encode polypeptide fragments of the present invention may then be used to obtain larger sequences using hybridization library screening techniques. For example a PCR clone may be labelled with radioactive atoms and used to screen a cDNA or genomic library from other species, preferably other plant species or fungal species. Hybridization conditions will typically be conditions of medium to high stringency (for example 0.03M sodium chloride and 0.03M sodium citrate at from about 50°C to about 60°C).

Degenerate nucleic acid probes encoding all or part of the amino acid sequence may also be used to probe cDNA and/or genomic libraries from other species, preferably other plant species or fungal species. However, it is preferred to carry out PCR techniques initially to obtain a single sequence for use in further screening procedures.

Polynucleotide sequences of the present invention obtained using the techniques described above may be used to obtain further homologous sequences and variants using the techniques described above. They may also be modified for use in expressing the polypeptides of the present invention in a variety of host cells systems, for example to optimise codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction enzyme recognition sites, or to alter the property or function of the polypeptides encoded by the polynucleotides.

Polynucleotides of the present invention may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40 nucleotides in length, and are also encompassed by the term polynucleotides of the present invention as used herein.

Polynucleotides or primers of the present invention may carry a revealing label. Suitable labels include radioisotopes such as ^{32}P or ^{35}S, enzyme labels, or other protein labels such as biotin. Such labels may be added to polynucleotides or primers of the present invention and may be detected using by techniques known *per se*.

Polynucleotides such as a DNA polynucleotide and primers according to the present invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques.

In general, primers will be produced by synthetic means, involving a step wise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

Longer polynucleotides will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. This will involve

making a pair of primers (e.g. of about 15-30 nucleotides) to a region of the endo-β-1,4-xylanase inhibitor gene which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from a fungal, plant or prokaryotic cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

Regulatory Sequences

Preferably, the polynucleotide of the present invention is operably linked to a regulatory sequence which is capable of providing for the expression of the coding sequence, such as by the chosen host cell. By way of example, the present invention covers a vector comprising the polynucleotide of the present invention operably linked to such a regulatory sequence, i.e. the vector is an expression vector.

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences.

The term "regulatory sequences" includes promoters and enhancers and other expression regulation signals.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site.

Enhanced expression of the polynucleotide encoding the polypeptide of the present invention may also be achieved by the selection of heterologous regulatory regions, e.g. promoter, secretion leader and terminator regions, which serve to increase expression

and, if desired, secretion levels of the protein of interest from the chosen expression host and/or to provide for the inducible control of the expression of the polypeptide of the present invention

Preferably, the nucleotide sequence of the present invention may be operably linked to at least a promoter.

Aside from the promoter native to the gene encoding the polypeptide of the present invention, other promoters may be used to direct expression of the polypeptide of the present invention. The promoter may be selected for its efficiency in directing the expression of the polypeptide of the present invention in the desired expression host.

In another embodiment, a constitutive promoter may be selected to direct the expression of the desired polypeptide of the present invention. Such an expression construct may provide additional advantages since it circumvents the need to culture the expression hosts on a medium containing an inducing substrate.

Examples of strong constitutive and/or inducible promoters which are preferred for use in fungal expression hosts are those which are obtainable from the fungal genes for xylanase (*xln*A), phytase, ATP-synthetase, subunit 9 (*oli*C), triose phosphate isomerase (*tpi*), alcohol dehydrogenase (*Adh*A), α-amylase (*amy*), amyloglucosidase (AG - from the *gla*A gene), acetamidase (*amd*S) and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoters.

Examples of strong yeast promoters are those obtainable from the genes for alcohol dehydrogenase, lactase, 3-phosphoglycerate kinase and triosephosphate isomerase.

Examples of strong bacterial promoters are the α-amylase and *SP02* promoters as well as promoters from extracellular protease genes.

Hybrid promoters may also be used to improve inducible regulation of the expression construct.

The promoter can additionally include features to ensure or to increase expression in a suitable host. For example, the features can be conserved regions such as a Pribnow Box or a TATA box. The promoter may even contain other sequences to affect (such as to maintain, enhance, decrease) the levels of expression of the nucleotide sequence of the present invention. For example, suitable other sequences include the Sh1-intron or an ADH intron. Other sequences include inducible elements - such as temperature, chemical, light or stress inducible elements. Also, suitable elements to enhance transcription or translation may be present. An example of the latter element is the TMV 5' signal sequence (see Sleat Gene 217 [1987] 217-225; and Dawson Plant Mol. Biol. 23 [1993] 97).

Secretion

Often, it is desirable for the polypeptide of the present invention to be secreted from the expression host into the culture medium from where the polypeptide of the present invention may be more easily recovered. According to the present invention, the secretion leader sequence may be selected on the basis of the desired expression host. Hybrid signal sequences may also be used with the context of the present invention.

Typical examples of heterologous secretion leader sequences are those originating from the fungal amyloglucosidase (AG) gene (*glaA* - both 18 and 24 amino acid versions e.g. from *Aspergillus*), the a-factor gene (yeasts e.g. *Saccharomyces* and *Kluyveromyces*) or the α-amylase gene (*Bacillus*).

Constructs

The term "construct" - which is synonymous with terms such as "conjugate", "cassette" and "hybrid" - includes the nucleotide sequence according to the present invention directly or indirectly attached to a promoter. An example of an indirect attachment is the provision of a suitable spacer group such as an intron sequence, such as the Sh1-intron or the ADH intron, intermediate the promoter and the nucleotide sequence of the present invention. The same is true for the term "fused" in relation to the present invention which includes

direct or indirect attachment. In each case, the terms do not cover the natural combination of the nucleotide sequence coding for the protein ordinarily associated with the wild type gene promoter and when they are both in their natural environment.

The construct may even contain or express a marker which allows for the selection of the genetic construct in, for example, a bacterium, preferably of the genus Bacillus, such as *Bacillus subtilis*, or plants, such as potatoes, sugar beet etc., into which it has been transferred. Various markers exist which may be used, such as for example those encoding mannose-6-phosphate isomerase (especially for plants) or those markers that provide for antibiotic resistance - e.g. resistance to G418, hygromycin, bleomycin, kanamycin and gentamycin.

Preferably the construct of the present invention comprises at least the nucleotide sequence of the present invention operably linked to a promoter.

Vectors

The term "vector" includes expression vectors and transformation vectors and shuttle vectors.

The term "expression vector" means a construct capable of *in vivo* or *in vitro* expression.

The term "transformation vector" means a construct capable of being transferred from one entity to another entity - which may be of the species or may be of a different species. If the construct is capable of being transferred from one species to another - such as from an *E.coli* plasmid to a bacterium, preferably of the genus Bacillus, then the transformation vector is sometimes called a "shuttle vector". It may even be a construct capable of being transferred from an *E.coli* plasmid to an Agrobacterium to a plant.

The vectors of the present invention may be transformed into a suitable host cell as described below to provide for expression of a polypeptide of the present invention. Thus, in a further aspect the invention provides a process for preparing polypeptides

according to the present invention which comprises cultivating a host cell transformed or transfected with an expression vector as described above under conditions to provide for expression by the vector of a coding sequence encoding the polypeptides, and recovering the expressed polypeptides.

The vectors may be for example, plasmid, virus or phage vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter.

The vectors of the present invention may contain one or more selectable marker genes. The most suitable selection systems for industrial micro-organisms are those formed by the group of selection markers which do not require a mutation in the host organism. Examples of fungal selection markers are the genes for acetamidase (*amdS*), ATP synthetase, subunit 9 (*oliC*), orotidine-5'-phosphate-decarboxylase (*pyrA*), phleomycin and benomyl resistance (benA). Examples of non-fungal selection markers are the bacterial G418 resistance gene (this may also be used in yeast, but not in fungi), the ampicillin resistance gene (*E. coli*), the neomycin resistance gene (*Bacillus*) and the *E.coli uidA* gene, coding for β-glucuronidase (GUS).

Vectors may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell.

Thus, polynucleotides of the present invention can be incorporated into a recombinant vector (typically a replicable vector), for example a cloning or expression vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention provides a method of making polynucleotides of the present invention by introducing a polynucleotide of the present invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells are described below in connection with expression vectors.

Tissue

The term "tissue" as used herein includes tissue *per se* and organ.

Host Cells

The term "host cell" - in relation to the present invention includes any cell that could comprise the nucleotide sequence coding for the recombinant protein according to the present invention and/or products obtained therefrom, wherein a promoter can allow expression of the nucleotide sequence according to the present invention when present in the host cell.

Thus, a further embodiment of the present invention provides host cells transformed or transfected with a polynucleotide of the present invention. Preferably said polynucleotide is carried in a vector for the replication and expression of said polynucleotides. The cells will be chosen to be compatible with the said vector and may for example be prokaryotic (for example bacterial), fungal, yeast or plant cells.

The gram-negative bacterium *E. coli* is widely used as a host for heterologous gene expression. However, large amounts of heterologous protein tend to accumulate inside the cell. Subsequent purification of the desired protein from the bulk of *E.coli* intracellular proteins can sometimes be difficult.

In contrast to *E.coli*, bacteria from the genus *Bacillus* are very suitable as heterologous hosts because of their capability to secrete proteins into the culture medium. Other bacteria suitable as hosts are those from the genera *Streptomyces* and *Pseudomonas*.

Depending on the nature of the polynucleotide encoding the polypeptide of the present invention, and/or the desirability for further processing of the expressed protein, eukaryotic hosts such as yeasts or fungi may be preferred. In general, yeast cells are preferred over fungal cells because they are easier to manipulate. However, some proteins are either poorly secreted from the yeast cell, or in some cases are not

processed properly (e.g. hyperglycosylation in yeast). In these instances, a fungal host organism should be selected.

Examples of preferred expression hosts within the scope of the present invention are fungi such as *Aspergillus* species (such as those described in EP-A-0184438 and EP-A-0284603) and *Trichoderma* species; bacteria such as *Bacillus* species (such as those described in EP-A-0134048 and EP-A-0253455), *Streptomyces* species and *Pseudomonas* species; and yeasts such as *Kluyveromyces* species (such as those described in EP-A-0096430 and EP-A-0301670) and *Saccharomyces* species.

Typical expression hosts may be selected from *Aspergillus niger*, *Aspergillus niger var. tubigenis*, *Aspergillus niger var. awamori*, *Aspergillus aculeatis*, *Aspergillus nidulans*, *Aspergillus orvzae*, *Trichoderma reesei*, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Kluyveromyces lactis* and *Saccharomyces cerevisiae*.

Organism

The term "organism" in relation to the present invention includes any organism that could comprise the nucleotide sequence coding for the recombinant protein according to the present invention and/or products obtained therefrom, wherein a promoter can allow expression of the nucleotide sequence according to the present invention when present in the organism. For the xylanase inhibitor aspect of the present invention, preferable organisms may include a fugus, yeast or a plant. For the xylanase aspect of the present invention, a preferabe organism may be a bacterium, preferably of the genus Bacillus, more preferably *Bacillus subtilis*.

The term "transgenic organism" in relation to the present invention includes any organism that comprises the nucleotide sequence coding for the protein according to the present invention and/or products obtained therefrom, wherein the promoter can allow expression of the nucleotide sequence according to the present invention within the organism. Preferably the nucleotide sequence is incorporated in the genome of the organism.

The term "transgenic organism" does not cover the native nucleotide coding sequence according to the present invention in its natural environment when it is under the control of its native promoter which is also in its natural environment. In addition, the present invention does not cover the native protein according to the present invention when it is in its natural environment and when it has been expressed by its native nucleotide coding sequence which is also in its natural environment and when that nucleotide sequence is under the control of its native promoter which is also in its natural environment.

Therefore, the transgenic organism of the present invention includes an organism comprising any one of, or combinations of, the nucleotide sequence coding for the amino acid sequence according to the present invention, constructs according to the present invention (including combinations thereof), vectors according to the present invention, plasmids according to the present invention, cells according to the present invention, tissues according to the present invention or the products thereof. The transformed cell or organism could prepare acceptable quantities of the desired compound which would be easily retrievable from, the cell or organism.

Transformation of Host Cells/Host Organisms

As indicated earlier, the host organism can be a prokaryotic or a eukaryotic organism. Examples of suitable prokaryotic hosts include *E. coli* and *Bacillus subtilis*. Teachings on the transformation of prokaryotic hosts is well documented in the art, for example see Sambrook et al (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring Harbor Laboratory Press) and Ausubel *et al.*, Current Protocols in Molecular Biology (1995), John Wiley & Sons, Inc.

If a prokaryotic host is used then the nucleotide sequence may need to be suitably modified before transformation - such as by removal of introns.

As mentioned above, a preferred host organism is of the genus Bacillus, such as *Bacillus subtilis*.

In another embodiment the transgenic organism can be a yeast. In this regard, yeast have also been widely used as a vehicle for heterologous gene expression. The species *Saccharomyces cerevisiae* has a long history of industrial use, including its use for heterologous gene expression. Expression of heterologous genes in Saccharomyces cerevisiae has been reviewed by Goodey et al (1987, Yeast Biotechnology, D R Berry et al, eds, pp 401-429, Allen and Unwin, London) and by King et al (1989, Molecular and Cell Biology of Yeasts, E F Walton and G T Yarronton, eds, pp 107-133, Blackie, Glasgow).

For several reasons *Saccharomyces cerevisiae* is well suited for heterologous gene expression. First, it is non-pathogenic to humans and it is incapable of producing certain endotoxins. Second, it has a long history of safe use following centuries of commercial exploitation for various purposes. This has led to wide public acceptability. Third, the extensive commercial use and research devoted to the organism has resulted in a wealth of knowledge about the genetics and physiology as well as large-scale fermentation characteristics of *Saccharomyces cerevisiae*.

A review of the principles of heterologous gene expression in Saccharomyces cerevisiae and secretion of gene products is given by E Hinchcliffe E Kenny (1993, "Yeast as a vehicle for the expression of heterologous genes", Yeasts, Vol 5, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.).

Several types of yeast vectors are available, including integrative vectors, which require recombination with the host genome for their maintenance, and autonomously replicating plasmid vectors.

In order to prepare the transgenic Saccharomyces, expression constructs are prepared by inserting the nucleotide sequence of the present invention into a construct designed for expression in yeast. Several types of constructs used for heterologous expression have been developed. The constructs contain a promoter active in yeast fused to the nucleotide sequence of the present invention, usually a promoter of yeast origin, such as the GAL1 promoter, is used. Usually a signal sequence of yeast origin, such as the sequence

encoding the SUC2 signal peptide, is used. A terminator active in yeast ends the expression system.

For the transformation of yeast several transformation protocols have been developed. For example, a transgenic Saccharomyces according to the present invention can be prepared by following the teachings of Hinnen et al (1978, Proceedings of the National Academy of Sciences of the USA 75, 1929); Beggs, J D (1978, Nature, London, 275, 104); and Ito. H et al (1983, J Bacteriology 153, 163-168).

The transformed yeast cells are selected using various selective markers. Among the markers used for transformation are a number of auxotrophic markers such as LEU2, HIS4 and TRP1, and dominant antibiotic resistance markers such as aminoglycoside antibiotic markers, eg G418.

Another host organism is a plant. The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material.

Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27).

Thus, in one aspect, the present invention relates to a vector system which carries a nucleotide sequence or construct according to the present invention and which is capable of introducing the nucleotide sequence or construct into the genome of an organism. such as a plant.

The vector system may comprise one vector, but it can comprise two vectors. In the case of two vectors, the vector system is normally referred to as a binary vector system. Binary

vector systems are described in further detail in Gynheung An et al. (1980), Binary Vectors. Plant Molecular Biology Manual A3, 1-19.

One extensively employed system for transformation of plant cells with a given nucleotide sequence is based on the use of a Ti plasmid from Agrobacterium tumefaciens or a Ri plasmid from Agrobacterium rhizogenes An et al. (1986), Plant Physiol. 81, 301-305 and Butcher D.N. et al. (1980), Tissue Culture Methods for Plant Pathologists, eds.: D.S. Ingrams and J.P. Helgeson, 203-208.

Several different Ti and Ri plasmids have been constructed which are suitable for the construction of the plant or plant cell constructs described above. A non-limiting example of such a Ti plasmid is pGV3850.

The nucleotide sequence or construct of the present invention should preferably be inserted into the Ti-plasmid between the terminal sequences of the T-DNA or adjacent a T-DNA sequenee so as to avoid disruption of the sequences immediately surrounding the T-DNA borders, as at least one of these regions appear to be essential for insertion of modified T-DNA into the plant genome.

As will be understood from the above explanation, if the organism is a plant, then the vector system of the present invention is preferably one which contains the sequences necessary to infect the plant (e.g. the vir region) and at least one border part of a T-DNA sequence, the border part being located on the same vector as the genetic construct. Preferably, the vector system is an Agrobacterium tumefaciens Ti-plasmid or an Agrobacterium rhizogenes Ri-plasmid or a derivative thereof, as these plasmids are well-known and widely employed in the construction of transgenic plants, many vector systems exist which are based on these plasmids or derivatives thereof.

In the construction of a transgenic plant the nucleotide sequence or construct of the present invention may be first constructed in a microorganism in which the vector can replicate and which is easy to manipulate before insertion into the plant. An example of a useful microorganism is *E. coli.*, but other microorganisms having the above properties may be

used. When a vector of a vector system as defined above has been constructed in E. coli. it is transferred, if necessary, into a suitable Agrobacterium strain, e.g. Agrobacterium tumefaciens. The Ti-plasmid harbouring the nucleotide sequence or construct of the present invention is thus preferably transferred into a suitable Agrobacterium strain, e.g. A. tumefaciens, so as to obtain an Agrobacterium cell harbouring the nucleotide sequence or construct of the present invention, which DNA is subsequently transferred into the plant cell to be modified.

In this way, the nucleotide or construct of the present invention can be introduced into a suitable restriction position in the vector. The contained plasmid is used for the transformation in *E.coli*. The *E.coli* cells are cultivated in a suitable nutrient medium and then harvested and lysed. The plasmid is then recovered. As a method of analysis there is generally used sequence analysis, restriction analysis, electrophoresis and further biochemical-molecular biological methods. After each manipulation, the used DNA sequence can be restricted and connected with the next DNA sequence. Each sequence can be cloned in the same or different plasmid.

After each introduction method of the desired nucleotide sequence according to the present invention in the plants the presence and/or insertion of further DNA sequences may be necessary. If, for example, for the transformation the Ti- or Ri-plasmid of the plant cells is used, at least the right boundary and often however the right and the left boundary of the Ti- and Ri-plasmid T-DNA, as flanking areas of the introduced genes, can be connected. The use of T-DNA for the transformation of plant cells has been intensively studied and is described in EP-A-120516; Hoekema, in: The Binary Plant Vector System Offset-drukkerij Kanters B.B., Alblasserdam, 1985, Chapter V; Fraley, et al., Crit. Rev. Plant Sci., 4:1-46; and An et al., EMBO J. (1985) 4:277-284.

Direct infection of plant tissues by Agrobacterium is a simple technique which has been widely employed and which is described in Butcher D.N. et al. (1980), Tissue Culture Methods for Plant Pathologists, eds.: D.S. Ingrams and J.P. Helgeson, 203-208. For further teachings on this topic see Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27). With

this technique, infection of a plant may be done on a certain part or tissue of the plant, i.e. on a part of a leaf, a root, a stem or another part of the plant.

Typically, with direct infection of plant tissues by Agrobacterium carrying the nucleotide sequence, a plant to be infected is wounded, e.g. by cutting the plant with a razor or puncturing the plant with a needle or rubbing the plant with an abrasive. The wound is then inoculated with the Agrobacterium. The inoculated plant or plant part is then grown on a suitable culture medium and allowed to develop into mature plants.

When plant cells are constructed, these cells may be grown and maintained in accordance with well-known tissue culturing methods such as by culturing the cells in a suitable culture medium supplied with the necessary growth factors such as amino acids, plant hormones, vitamins, etc. Regeneration of the transformed cells into genetically modified plants may be accomplished using known methods for the regeneration of plants from cell or tissue cultures, for example by selecting transformed shoots using an antibiotic and by subculturing the shoots on a medium containing the appropriate nutrients, plant hormones, etc.

Further teachings on plant transformation may be found in EP-A-0449375.

Production of the Polypeptide

According to the present invention, the production of the polypeptide of the present invention can be effected by the culturing of, for example, microbial expression hosts, which have been transformed with one or more polynucleotides of the present invention, in a conventional nutrient fermentation medium. The selection of the appropriate medium may be based on the choice of expression hosts and/or based on the regulatory requirements of the expression construct. Such media are well-known to those skilled in the art. The medium may, if desired, contain additional components favouring the transformed expression hosts over other potentially contaminating microorganisms.

Antibodies

The amino acid sequence of the present invention can also be used to generate antibodies - such as by use of standard techniques - against the amino acid sequence.

For the production of antibodies, various hosts including goats, rabbits, rats, mice, etc. may be immunized by injection with the inhibitor or any portion, variant, homologue, fragment or derivative thereof or oligopeptide which retains immunogenic properties. Depending on the host species, various adjuvants may be used to increase immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminium hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. BCG (*Bacilli Calmette-Guerin*) and *Corynebacterium parvum* are potentially useful human adjuvants which may be employed.

Monoclonal antibodies to the amino acid sequence may be even prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique originally described by Koehler and Milstein (1975 Nature 256:495-497), the human B-cell hybridoma technique (Kosbor *et al* (1983) Immunol Today 4:72; Cote *et al* (1983) Proc Natl Acad Sci 80:2026-2030) and the EBV-hybridoma technique (Cole *et al* (1985) Monoclonal Antibodies and Cancer Therapy, Alan R Liss Inc, pp 77-96). In addition, techniques developed for the production of "chimeric antibodies", the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity can be used (Morrison *et al* (1984) Proc Natl Acad Sci 81:6851-6855; Neuberger *et al* (1984) Nature 312:604-608; Takeda *et al* (1985) Nature 314:452-454). Alternatively, techniques described for the production of single chain antibodies (US-A-4946779) can be adapted to produce inhibitor specific single chain antibodies.

Antibodies may also be produced by inducing *in vivo* production in the lymphocyte population or by screening recombinant immunoglobulin libraries or panels of highly specific binding reagents as disclosed in Orlandi *et al* (1989, Proc Natl Acad Sci 86: 3833-3837), and Winter G and Milstein C (1991; Nature 349:293-299).

PROTOCOLS

PROTOCOL 1

XYLANASE ASSAY

(Endo-β-1,4-Xylanase activity)

Xylanase samples are diluted in citric acid (0.1M) - *di*-sodium-hydrogen phosphate (0.2M) buffer, pH 5.0, to obtain approx. OD = 0.7 in the final assay. Three dilutions of the sample and an internal standard with a defined activity are thermostated for 5 minutes at 40°C. To time = 5 minutes, 1 Xylazyme tab (crosslinked, dyed xylan substrate) is added to the enzyme solution. To time = 15 minutes the reaction is terminated, by adding 10 ml of 2% TRIS. The reaction mixture is centrifuged and the OD of the supernatant is measured at 590nm. Taking into account the dilutions and the amount of xylanase, the activity (TXU, Total-Xylanase-Units) of the sample can be calculated relatively to the standard.

PROTOCOL 2

STICKINESS PROTOCOL

(Stickiness Determination)

Dough stickiness is measured on a TA-XT2 system (Stable Micro Systems) using a SMS Dough Stickiness Cell. The protocol is a modified version of the method described by Chen and Hoseney (1995). A dough is made from flour, 2% NaCl and water to 400 Brabender Units (BU) using a Farinograph (AACC method 54-21). The flour and NaCl is dry mixed for 1 minute. Water is added and the dough is mixed for another 5 minutes. The obtained dough could advantageously be rested for 10, 30 or 45 minutes in sealed containers at 30°C.

Approx. 4 gram dough is placed in the Dough Stickiness Cell. 4 mm dough is extruded to obtain an uniform extrusion. Hereafter 5 measurement are made according to Stable Micro Systems protocol (TA-XT2 application study for measurement of dough stickiness). In brief, 1mm dough is extruded. The probe (25 mm perspex cylinder probe), connected to the TA-XT2 system, is pressed into the extruded dough at a set force. The probe is raised and the adhesion between the dough and the probe is recorded. The following TA-XT2 setting are used:

Option:	Adhesive test
Pre-test speed:	2.0 mm/s
Test speed:	2.0 mm/s
Post-test speed:	10.0 mm/s
Distance:	15 mm
Force:	40 g
Time:	0.1 s
Trigger Type:	Auto - 5 g
Data Acquisition rate:	400 pps

The results recorded form the test are peak force, meaning the force needed to raise the probe from the extruded dough. The distance, meaning the distance the dough attach to the probe. Area, meaning force multiplied by distance.

Dough stickiness is depending on the quality of the flour used and the recipe. Therefore a non - sticky dough is a dough differing in stickiness from 100% to 200% (relative) compared to a reference dough, without the xylanase or having preferably less 70% (relative) of the stickiness obtained with a commercial fungal xylanase (i.e. Pentopan mono BG, Novo) when dosed at a levels giving the same volume increase in a baking trial.

PROTOCOL 3

INHIBITOR ASSAY PROTOCOL

(Inhibitor assay)

To detect the inhibitor during isolation and characterisation the following assay is used. 100 µl inhibitor fraction, 250 µl xylanase solution (containing 12 TXU/ml) and 650 µl buffer (0.1 M citric acid - 0.2M *di*-sodium hydrogen phosphate buffer, pH 5.0) is mixed. The mixture is thermostated for 5 minutes at 40.0°C. At time = 5 minutes one Xylazyme tab is added. At time = 15 minutes the reaction is terminated by adding 10 ml 2% TRIS. The reaction mixture is centrifuged (3500 g, 10 minutes, room temperature) and the supernatant is measured at 590 nm. The inhibition is calculated as residual activity compared to the blank.

The blank is prepared the same way, except that the 100 µl inhibitor is substituted with 100 µl buffer (0.1 M citric acid - 0.2 M *di*-sodium hydrogen phosphate buffer, pH 5.0).

PROTOCOL 4

GLUCANASE PROTOCOL

(Endo-β-1,4-Glucanase activity)

Glucanase samples are diluted in 0.1M sodium-acetate - citric acid buffer, pH = 5.0, to obtain approx. OD = 0.7 in the final assay. Three dilutions of the sample and an internal standard with a defined activity are thermostated for 5 minutes at 40°C. To time = 5 minutes, 1 Glucazyme tab (crosslinked, dyed glucan substrate) is added to the enzyme solution. To time = 15 minutes the reaction is terminated, by adding 10 ml of 2% TRIS. The reaction mixture is centrifuged and the OD of the supernatant is measured at 590nm. Taking into account the dilutions and the amount of glucanase, the activity (BGU, Beta-Glucanase-Units) of the sample can be calculated relatively to the standard.

SUMMARY

In summary the present invention provides *inter alia*:

a. The isolation of an endogenous endo-β-1,4-xylanase inhibitor from wheat flour.

b. The characterisation of an endogenous endo-β-1,4-xylanase inhibitor isolated from wheat flour.

c. The characterisation of the effect of endogenous endo-β-1,4-xylanase inhibitor on different xylanases.

d. A means for selecting xylanases not detrimentally affected by endogenous endo-β-1,4-xylanase inhibitor.

e. A means for selecting xylanases which are not detrimentally affected by endo-β-1,4-xylanase inhibitors.

f. Xylanases that provide dough exhibiting favourable volume and acceptable stickiness than when compared to doughs comprising fungal xylanases.

g. A method for screening xylanases and/or mutating the same using an endogenous endo-β-1,4-xylanase inhibitor, and the use of those xylanases or mutants thereof in the manufacture of doughs.

h. A foodstuff prepared with the xylanases of the present invention.

DEPOSITS

The following samples were deposited in accordance with the Budapest Treaty at the recognised depositary The National Collections of Industrial and Marine Bacteria Limited (NCIMB) at 23 St. Machar Drive, Aberdeen, Scotland, United Kingdom, AB2 1RY on 22 December 1998:

DH5α - - PCR2.1/BSxylanase	NCIMB number NCIMB 40999
BL21"DH3" - - PET24A+/XM1	NCIMB number NCIMB 41000
BL21"DH3" - - PET24A+/XM2	NCIMB number NCIMB 41001

DH5α - - PCR2.1/BSxylanase comprises wild type xylanase.
BL21"DH3" - - PET24A+/XM1 comprises XM1 xylanase.
BL21"DH3" - - PET24A+/XM2 comprises XM1 xylanase.

The present invention also encompasses sequences derivable and/or expressable from those deposits and embodiments comprising the same.

INTRODUCTION TO THE EAMPLES SECTION AND THE FIGURES

The present invention will now be described, by way of example only, with reference to the accompanying drawings in which:-

Figure 1 shows a graph;

Figure 2 shows a graph;

Figure 3 shows a graph;

Figure 4 shows a graph;

Figure 5 shows a graph;

Figure 6 shows a graph;

Figure 7 shows a graph;

Figure 8 shows a graph;

Figure 9 shows an image result of an SDS PAGE experiment;

Figure 10 shows a graph;

Figure 11 shows a graph;

Figure 12 shows a graph;

Figure 13 shows a graph;

Figure 14 shows a graph;

Figure 15 shows a graph;

Figure 16 shows a graph;

Figure 16 shows a graph;

Figure 17 shows a graph;

Figure 18 shows a graph;

Figure 19 shows a graph;

Figure 20 shows a graph;

Figure 21 shows a graph;

Figure 22 shows a graph; and

Figure 23 shows a graph.

In slightly more detail:

Figure.1. Stickiness as a function of xylanases, dose and resting time.

Figure 2. Stickiness as a function of xylanases, dose and resting time.

Figure. 3. Gel filtration chromatography of a 75 ml inhibitor extract sample. Column: 500 ml Superdex G-25 F. Flow: 10 ml/min. Fraction size: 30 ml.

Figure 4. Cation exchange chromatography of a 240ml gel filtrated inhibitor extract sample. Column: 50 ml Sepharose SP, Flow: 5.0 ml/min, Fraction size: 10ml.

Figure 5. HIC chromatography of a 147 ml ion exchanged inhibitor extract sample added $(NH_4)_2SO_4$ to 1.0M. Column: 10 ml Phenyl HIC, Flow: 2.0 ml/min, Fraction size: 2.5 ml.

Figure. 6. Preparative gel filtration chromatography of 2 ml concentrated inhibitor sample. Inhibitor eluted at 176 ml. Column: 330 ml Superdex 75 PG (Pharmacia). Eluent: 50 mM NaOAc, 200 mM NaCl, pH 5.0. Flow: 1 ml/minute. Fraction size: 5.5 ml

Figure 7. Analytical gel filtration chromatography of 100 µl concentrated inhibitor sample. Inhibitor eluted at 10.81 ml. Column: 24 ml Superdex 75 10/30 (Pharmacia, Sweden). Eluent: 50 mM NaOAc, 100 mM NaCl, pH 5.0, Flow: 0.5 ml/minute. Fraction size: 2.0 ml.

Figure 8. Log(MW) as function of Kav for standard proteins run on a Superdex 75 10/30.

Figure 9. SDS PAGE of fraction 31, 32 and fraction 33 from Preparative gel filtration. Lane 1 and 3 are MW markers (Pharmacia's LMW markers, Sweden). Lane 2 and 4 are

frac. 32, loaded with 10 and 25 µl respectively. Lane 6 and 8 are frac. 31, loaded with 10 and 25. Lane 7 and 9 are frac. 33, loaded with 10 and 25.

Figure 10. Cation exchange chromatogram of pure xylanase + boiled inhibitor extract. Sample: 1 ml desalted 980601 + boiled inhibitor extract. Column: 1 ml Source S 15. Buffer system: A: 50 mM NaOAc, pH 4.5, B: A + 1 M NaCl. Flow: 2 ml/minute.

Figure 11. Cation exchange chromatogram of pure xylanase after three hours incubation with inhibitor extract. Sample: 1 ml desalted 980601 + inhibitor. Column: 1 ml Source S 15. Buffer system: A: 50 mM NaOAc, pH 4.5, B: A + 1 M NaCl. Flow: 2 ml/minute.

Fig. 12. Residual activity, % of four xylanases as a function of inhibitor concentration. The four xylanases used are: ---- X1, ---- X3, ----*BX.*, -----Novo.

Figure 13. Residual activity of 980601 (coli-1), 980603 (Belase) and three mutants of 980601 (XM1, XM2 and XM3) after incubation with a flour extract..

Figure 14. Line-weaver - Burk plot of xylanase (980601) +/- inhibitor. Substrate concentration is % azo-xylan. V is relative OD 590 from assay (where 100 is S=2%).

Figure 15. Inhibition of three xylanases (980601 = *Bac. sub.* wt, 980801 = X1 and 980901 = *Thermomyces*) as a function of pH. The data are obtained by substrating relevant blanks.

Figure 16. pH optimum for three xylanases (980601 = BX, 980801 = X1 and 980901 = Novo).

Figure 17. Spec. vol = f(xylanase x dose)

Figure 18. Spec. vol. increase = f(xylanase x dose)

Figure 19. Stickiness = f(xylanase x dose)

Figure 20. Stickiness as function of different xylanase preparations and control, measured after 10 (_10) and 45 (_45) minutes resting. 980603 is purified Röhm xylanase, XM1 is xylanase mutant 1 and #2199 is Röhm's Veron Special product.

Figure 21. Stickiness increase as function of three xylanase preparations, after 10 (_10) and 45 (_45) minutes resting. 980603 is purified Röhm xylanase. XM1 is xylanase mutant 1 and #2199 is Röhm's Veron Special product.

Figure 22. Stickiness increase as function of two xylanase preparations, after 10 (_10) and 45 (_45) minutes resting. XM1 is xylanase mutant 1 and #2199 is Röhm's Veron Special product.

Figure 23. Stickiness increase as function of added Endo-β-1,4-Glucanase. 1: Control dough without xylanase, 2: 7500 TXU pure Röhm xylanase/kg flour, 3: 7500 TXU pure Röhm xylanase/kg flour + 158 BGU/kg Flour, 4: 15000 TXU pure Röhm xylanase/kg flour, 5: 15000 TXU pure Röhm xylanase/kg flour + 316 BGU/kg Flour. Dough were measured after 10 (Stik_10) and 45 (Stik_45) minutes.

EXAMPLES

Example 1

Dough stickiness as a function of different xylanases, doses and resting time.

The following xylanases ability to give dough stickiness were tested.

(See also Chen, W. Z. and Hoseney, R. C. (1995). Development of an objective method for dough stickiness. Lebensmittel Wiss u.- Technol., 28, 467-473.)

Enzymes

X1 corresponds to a purified sample of endo-β-1,4-xylanase from *Aspergillus niger*. This xylanase has an activity of 8400 TXU (15000 TXU/mg).

Novo corresponds to a purified sample of Novo's Pentopan Mono BG from *Thermomyces*. This xylanase has an activity of 350.000 TXU (56000 TXU/mg).

BX corresponds to a purified sample of the new bacterial xylanase. This sample has an activity of 2000 TXU (25000 TXU/mg).

Röhm corresponds to Röhm's bacterial xylanase, Veron Speciel. This sample has an activity of 10500 TXU (25000 TXU/mg).

Xylanase Assay

Xylanase assays were performed according to Protocol 1

Flour

Two kinds of flour have been used in this trial:

Danish flour, batch no 98022 and German flour, batch no. 98048. The water absorbtions, at 400 BU, of the two kinds of flour are 58 and 60% respectively.

Dough preparation

Dough were prepared as described in protocol 2. After mixing the dough rested for 10 and 45 minutes respectively at 30°C in sealed containers.

Stickiness measurement

Stickiness measurements were performed according to Protocol 2

Results and discussion

Fungal xylanases versus new bacterial xylanase

The following dough were made and tested for dough stickiness after 10 and 45 minutes in flour 98048.

Table. 1

Dough made with different doses of two fungal xylanases and one bacterial xylanase. (Dose is calculated per kg of flour.)

Enzyme	TXU./kg
Blank	0
X1 (980801)	1500
	10000
Novo (#2165)	5000
	60000
BX (980802)	1500
	15000

The dough in Table 1 gave the dough stickiness results presented in Table 2 and Figure 1.

Table. 2

Dough made with different doses of different xylanases vs.blank.

The dough was rested for 10 and 45 minutes, respectively.

Stickiness is given as g x s, the stickiness figure is an average of 5 determinations.

Dough	Stickiness, g x s	Std.Dev	std.dev., %
Control, 10min	5.533	0.16	2.89

Control, 45min	8.103	0.277	3.42
1500 X1, 10min	7.275	0.204	2.80
1500 X1, 45min	8.675	0.134	1.54
10000 X1, 10min	9.295	0.802	8.63
10000 X1, 45min	13.339	1.264	9.48
5000 Novo, 10min	6.757	0.218	3.23
5000 Novo, 45min	7.23	0.337	4.66
60000 Novo, 10min	10.972	0.519	4.73
60000 Novo, 45min	16.559	1.626	9.82
1500 BX, 45min	4.372	0.358	8.19
15000 BX, 10 min	6.567	0.639	9.73
15000 BX, 45min	5.545	0.518	9.34

The data from Table 2 are illustrated in Figure 1.

As can be seen from Table 2 and Figure 1 the fungal xylanase X1 and the xylanase in Novo's product give rise to dough stickiness. The new bacterial xylanase does not give rise to the same stickiness. As a matter of fact, the stickiness seems to decrease compared with control.

New bacterial xylanase vs Röhm's bacterial xylanase

To test the functionality of the novel bacterial xylanase compared to the bacterial xylanase in Röhm's product: Veron Special, the following dough was made (see Table 3) using flour 98022.

Table 3

Dough made with different doses of two bacterial xylanases.

(Dose is calculated per kg of flour.)

Enzyme	TXU/kg
Blank	0
BX	5000
	15000
Röhm	5000
	15000

The dough in Table 3 gave the dough stickiness results presented in Table 4 and Figure 2.

Table 4

Dough made with different doses of different xylanases vs. blank.

Stickiness is given as g x s, the stickiness figure is an average of 5 determinations.

Dough	Stickiness, g x mm	Std.Dev	std.dev,. %
Control 10min	5.269	0.16	3.04
Control 45min	5.484	0.277	5.05
5000 BX, 10min	4.443	0.204	4.59
5000 BX, 45min	4.474	0.134	3.00
15000 BX, 10min	4.791	0.352	7.35
15000 BX, 45min	6.288	0.599	9.53
5000 Röhm,10min	5.077	0.218	4.29
5000 Röhm, 45min	6.757	0.337	4.99
15000 Röhm, 10min	7.749	0.519	6.70
15000 Röhm, 45min	10.98	0.907	8.26

The data from Table 4 are illustrated in Figure 2.

The results show that BX (the new bacterial xylanase) gives rise to much less stickiness than the fungal xylanase tested. Moreover, it is found that the new xylanase gives rise to much less dough stickiness than Röhm's bacterial xylanase.

Example 2

Inhibitor purification, characterisation and effect on xylanases

Wheat flour contains endogeneous endo-β-1,4-xylanase inhibitor. The inhibitor can be extracted from wheat flour by a simple extraction using water, meaning that the inhibitor is water-soluble. The inhibitor was purified using gel filtration-, ion exchange- and hydrophobic interaction chromatographic techniques. Characterisation of the purified inhibitor, using analytical gel filtration chromatography and SDS PAGE, revealed a

poly-peptide of approx. 34 KDa. The purified inhibitor was N-terminal sequenced. The N-terminal sequence revealed two peptides (57 and 38 aa's, respectively).

The preliminary experiment with the inhibitor indicated that the decrease in xylanase activity found could be due to proteolysis. However, analysis of incubation trials (xylanase + inhibitor) and kinetics on the inhibitor indicated that the observed decrease in xylanase activity was due to a competitive inhibitor.

Inhibitor experiments using several xylanases indicate differences in sensibility towards the inhibitor. Some xylanases are inhibited almost 100% by the inhibitor (at a lower inhibitor: xylanase ratio than present in the flour). By varying pH in the inhibitor assay it turns out the inhibition is highly dependent on the pH in the assay. Examining the xylanase mutants revealed that changing one amino acid can mean a 250% decrease in inhibition.

Flour

Two different kinds of flour was used in these experiments (batch 98002 and 98026). Flour batch 98002 is a Danish flour. Flour batch 98026 is German flour.

Inhibitor extraction

The inhibitor was extracted from the flour using ice cold distilled water and stirring. One equivalent of flour was added two equivalents of ice cold distilled water. The mix was added a magnetic bar, placed in an ice bath and stirred for 20 minutes. After stirring the flour slurry was poured into centrifuge vials and centrifuged (10000g, 4°C and 10 minutes). The supernatant contained the xylanase inhibitor.

Inhibitor assay

Inhibitor assays were performed according to Protocol 3

Inhibitor Assay II

PROTOCOL 5

To study kinetics on the inhibitor a soluble substrate was used (Azo-xylan, Megazyme). A 2% (w/v) solution of the substrate was prepared, according to manufacturers protocol, in 20 mM NaPi, pH 6.0. The assay was performed by pre-heating substrate, xylanase and inhibitor at 40°C for 5 minutes. The xylanase used was 980601, diluted to 40 TXU/ml and the inhibitor was extracted from flour 98002. 0.5 ml of substrate, 0.1 ml of xylanase and 0.1 ml of inhibitor was mixed at time = 0 minutes, 40°C. At time = 125 minutes, the reaction was terminated by adding 2 ml of ethanol (95%), followed by vortexing for 10 seconds. Precipitated unhydrolysed substrate was removed by centrifugation (3500 x g, 10 minutes, room temperature). OD in the supernatant was measured against water at 590 nm.

Blank was prepared the same way. The only modification was substitution of the inhibitor with 20 mM NaPi, pH 6.0.

For kinetic experiments with decreased substrate concentration, the following substrate concentrations were made by dilution in 20 mM NaPi, pH 6.0. 2%, 1%, 0.4% and 0.2% soluble azo-xylan (w/v).

Inhibitor isolation

After extraction of a 100 g flour sample (98026) the xylanase inhibitor was purified by the following chromatographic techniques:

Gel filtration chromatography (this procedure was run twice)

75 ml extract was applied to a 500 ml Superdex G-25 F (Pharmacia, Sweden) column at 10ml/minute, calibrated with 20 mM NaOAc, pH 4.25. Eluent was collected in 30 ml fractions at the same flow. All fractions were spotted for inhibitor.

Cation exchange chromatography (this procedure was run twice)

The inhibitor peak collected from the gel filtration run (240 ml) was applied to a 50 ml SP Sepharose (Pharmacia, Sweden) column at 5 ml/minute. After loading, the column was washed to baseline with A buffer (20mM NaOAc, pH 4.25). The inhibitor was eluted by a linear gradient from A to B buffer (B: A + 350mM NaCl) over 10 column volumes at the same flow. The eluate was collected in fractions of 10ml. Every second fraction was spotted for xylanase inhibitor.

Hydrophobic interaction chromatography (this procedure was run twice)

The inhibitor peak from the cation exchange chromatography (110 ml) was added $(NH_4)_2SO_4$ to 1.0 M and applied to a 10 ml Phenyl Sepharose HIC (Pharmacia, Sweden) column at 2 ml/minute. The inhibitor was eluted from the column by a 12 column volume linear gradient from A (20mM NaPi, 1M $(NH_4)_2SO_4$, pH 6.0) to B (20 mM NaPi, pH 6.0). The eluate was collected in fractions of 2.5 ml. Every second fraction was spotted for xylanase inhibitor.

Preparative gel filtration chromatography

5 ml inhibitor peak from HIC run was up-concentrated to 2 ml using a rotatory evaporator. This sample was loaded to a 330 ml Superdex 75 PG (Pharmacia, Sweden) column at 1 ml/minute. The buffer system used was 50 mM NaOAc, 0.2 M NaCl, pH 5.0. The eluate was collected in 5.5 ml fractions. Every second fraction was spotted for xylanase inhibitor.

Inhibitor characterisation

Analytical gel filtration chromatography

100 µl (concentrated two times on rotatory evaporator) of the inhibitor peak from the HIC run was applied to a 24 ml Superdex 75 10/30 (Pharmacia, Sweden) at 0.5 ml/minute. Running buffer used was 50 mM NaOAc, 0.1 M NaCl, pH 5.0. Eluate was collected in fractions of 2 ml. All fractions were spotted for inhibitor.

To be able to determine the size of the inhibitor a series of known proteins were applied to the 24 ml Superdex 75 10/30 column. The conditions for this run were as described above.

The standard proteins used were:

Protein	Size, KDa.
BSA	67
Ovalbumine	43
Chymotrypsine	25
Ribonuclease A	13.7

The proteins were detected at 280 nm.

SDS PAGE

Fractions from Preparative gel filtration chromatography were added SDS sample buffer (prepared according to NOVEX protocol), boiled for three minutes and loaded on a 8-16% PAGE gel (NOVEX). The gel was stained according to NOVEX's protocol for silver staining. As molecular weight markers, Pharmacia's LMW markers were used.

Inhibitor kinetics

See "Inhibitor Assay II".

N-terminal sequence

A 5 ml fraction (taken in the middle of the inhibitor peak) from the Preparative gel filtration chromatography was N-terminal sequenced.

Analysis of protease activity

To be able to determine whether the found inhibitor effect was due to an inhibitor or a protease hydrolysing the xylanase, the following experiments were carried out.

Incubation trials

2 ml of pure xylanase, 980601 (see Endo-β-1,4-xylanases) was incubated with 0.25 ml of inhibitor extract for three hours at 40°C. As a control the same incubation was made with boiled (5 minutes) inhibitor extract. After incubation the samples were added 50 mM NaOAc, pH 4.5 to 2.5 ml and desalted by gel filtration on a PD-10 column (Pharmacia, Sweden), obtaining 3.5 ml sample in 50 mM NaOAc, pH 4.5.

Analysis for hydrolysis

The two samples of pure xylanase from the incubation trials were analysed on a SOURCE 15 S column. 1 ml of the gel filtered sample was applied to the column (calibrated with A buffer: 50 mM NaOAc, pH 4.5) at 2 ml/minute. The sample was eluted with a linear gradient from A to B (B: A + 1 M NaCl) over 20 column volumes and collected in 2 ml fractions. The xylanase was detected using OD 280 nm and spotted for xylanase activity in the fractions (100 µl fraction + 900 µl buffer (0.1 M citric acid - 0.2 M *di*-sodium hydrogene phosphate buffer, pH 5.0) + 1 Xylazyme tab, 10 minutes, 40°C. Reaction terminated with 10 ml 2% TRIS, blue colour = xylanase activity).

Determination of inhibition as a function of pH

These experiments were carried out as described under "Inhibitor assay" with the following modifications. Besides using 650 µl buffer (0.1 M citric acid - 0.2 M *di*-sodium hydrogene phosphate) pH 5.0 in the assay, the assay was also carried out using the same buffer system at pH: 4, 6 and 7.

Endo-β-1,4-xylanases

The following xylanase preparations were used:

980601 (BX): Purified preparation of the new bacterial xylanase expressed in *E. coli*. (1225 TXU/ml)

980603 (Röhm): Purified preparation of Frimond's Belase xylanase (identical to Röhm's) (1050 TXU/ml)

980801 (X1): Purified X1 from *Aspergillus niger* (8400 TXU/g)

980802 (Röhm): Purified preparation of Frimond's Belase xylanse (identical to Röhm's) (265 TXU/ml)

980901 (Novo): Purified preparation of *Thermomyces* xylanase from Novo's Pentopan mono BG (2900 TXU/ml)

980903 (XM1): Purified mutant of *Bacillus sub.* wild type xylanase expressed in *E. coli*. (1375 TXU/ml)

980906 (XM3): Purified mutant of *Bacillus sub.* wild type xylanase expressed in *E. coli*. (1775 TXU/ml)

980907 (XM2): Purified mutant of *Bacillus sub.* wild type xylanase expressed in *E. coli*. (100 TXU/ml)

9535 (X3): Purified xylanase, X3 from *Aspergillus niger* (6490 TXU/ml)

Results and discussion

Inhibitor extraction for isolation and characterisation

100 g flour (98026) was extracted. After centrifugation a supernatant of 150 ml was obtained. The presence of inhibitor was checked in this extract (Table 5) and found positive.

Table 5. Residual activity as a function of +/- addition of inhibitor extract from wheat flour (98026). The xylanase used is 980601.

	- inhibitor	+inhibitor	Residual activity, %
OD 590	0.675	0.165	24.44

Inhibitor isolation

75 ml of the inhibitor extract was loaded on a 500 ml gel filtration column (Figure 3). After spotting for the inhibitor, it could be located in fractions [4 - 11] (Table 6).

Table 6. Fractions from gel filtration chromatography of 75 ml inhibitor extract assayed for xylanase inhibitor. OD run 1 respectively 2 correspond to the two runs that were performed on the column. Inhibitor was found present in fractions [4 - 11]. These fractions were pooled for each run, giving two times 240ml.

Fraction no.	OD run 1	OD run 2
1	0.674	
2	0.665	
3	0.652	
4	0.618	0.476
5	0.388	0.166
6	0.186	0.126
7	0.188	0.18
8	0.277	0.217
9	0.381	0.231
10	0.406	0.246
11	0.395	0.435
12	0.725	
13	0.683	
14	0.762	
15	0.737	

The pool of the inhibitor peak in both runs on the gel filtration column, was approx. 240 ml.

Two times, 240 ml pool from gelfiltration was applied to the cation exchanger. The flow through was found negative for inhibitor. As can be seen from Figure 4 and Table 7 the inhibitor bound to the column and eluted at approx. 750 mM NaCl.

Table 7. Fractions from cation exchange chromatography of 240 ml gel filtered inhibitor extract assayed for the presence of xylanase inhibitor. OD run 1 respectively 2 correspond to the two runs that were performed on the column. Inhibitor was found present in fractions [44 - 54].

Fraction no.	OD run 1	OD run 2
40	0.476	0.624
42	0.407	0.58
44	0.404	0.398
46	0.22	0.137
48	0.144	0.107
50	0.198	0.126
52	0.302	0.208
54	0.395	0.435
56	0.457	0.495
58	0.463	0.606

The pool of inhibitor from the ion exchange runs was 110 ml from each run. These two pooled fractions were added $(NH_4)_2SO_4$ to 1.0 M and applied to the HIC column in two runs. The flow through was spotted for inhibitor and found negative. As can be seen from Figure 5 and Table 8 all inhibitor bound to the column and a good separation was obtained.

The analysis of the fractions from the HIC chromatography is shown in Table 8.

Table. 8. Fractions from HIC chromatography of 147 ml inhibitor extract assayed for xylanase inhibitor. OD run 1 respectively 2 corresponds to the two runs that were performed on the column. Inhibitor was found present in fractions [15 - 23].

Fraction no.	OD run 1	OD run 2
Blank	0.469	0.659
12	0.462	0.622
14	0.486	0.555
16	0.202	0.188
18	0.1	0.118
20	0.102	0.146
22	0.242	0.193
24	0.392	0.502
26	0.485	0.6

Fractions 17 and 18 from the HIC chromatography were concentrated approx. two times and applied to a preparative gel filtration column (Figure 6).

The analysis of the fractions from the preparative gel filtration is shown in Table 9.

Table 9. Fractions from Preparative gel filtration chromatography of 2 ml concentrated inhibitor sample assayed for the presence of xylanase inhibitor. Inhibitor was found present in fractions [31 - 33].

Fraction no.	OD 280nm
26	0.738
28	0.774
30	0.645
32	0.117
34	0.705
36	0.749
38	0.754
40	0.761
42	0.769

Inhibitor characterisation

Analytical gel filtration chromatography

100 µl two times concentrated inhibitor sample from fraction 18 in the second HIC run was applied to a 24 ml analytical Superdex 75 10/30 (Pharmacia, Sweden) (Figure 7). The eluate was collected in fractions of 2 ml. These fractions were assayed for the xylanase inhibitor (Table 10).

Table 10. Fractions from analytical gel filtration chromatography of 100 µl concentrated inhibitor sample assayed for the presence of xylanase inhibitor. Inhibitor was found present in fractions [6 - 7].

Fraction no.	OD
Blank	0.613
6	0.233
7	0.304
8	0.51
9	0.569
10	0.565
11	0.652

After the gel filtration of the up-concentrated inhibitor sample a mix of four standard molecular weight proteins was applied to the column, using the exactly same procedure (chromatogram not shown). In Table 11 the molecular weights and the elution times for the proteins are summarised.

Table 11. Standard proteins used for determination of the MW of the inhibitor. Abbreviations and equations used are explained below the table.

Std. protein	Ve, ml	Kav*	MW, kDa	log (MW)
BSA	9.46	0.059508	67	1.826075
Ovalbumin	10.38	0.119017	43	1.633468
Chymotrypsin	12.49	0.255498	25	1.39794
Ribonuklease A	13.49	0.320181	13.7	1.136721

*) Kav = (Ve - Vo)/Vt - Vo)

Where: Ve = ret. Time, ml =
Vo = void vol., ml = 8.54
Vt = 24ml = 24

Plotting the log (MW) as a function of Kav. It is possible to obtain an equation and estimating the molecular size of an unknown molecule (Figure 8).

Using the equation obtained in figure 6 and the retention time for the inhibitor, it is possible to calculate the molecular size of the inhibitor:

$$\text{MW, kDa} = 10^{(-2,4485 \times \text{kav} + 1.9602)}$$

$$= 10^{(-2,4485 \times 0.173559 + 1.9602)}$$

$$= 10^{1.5352}$$

$$= 34.29$$

The molecular weight found for the inhibitor was higher than we expected according to Rouau and Surget (1998, Evidence for the presence of a Pentosanase Inhibitor in Wheat Flour. Journal of Cereal Science. 28: 63 - 70), the MW of the molecule is approx. 8 KDa. The MW obtained by gel filtration could be explained by aggregation of several inhibitor molecules. To study this further an SDS PAGE gel was run of fractions 31, 32 and 33 from the preparative gel filtration chromatography (Figure 9).

It is clear from the SDS PAGE that the xylanase inhibitor in fact has a molecular weight of approx. 34 KDa. It is also evident from the gel that there is a small band at approx. 40 KDa. So, the inhibitor preparation is not completely pure. This was verified during the N - terminal sequencing.

A sample of approx. 5 ml fraction 32 from preparative gel filtration of the inhibitor was sequence analysed. To be able to determine whether a sequence analysis was possible, the following calculations and assumption were made, to determine the amount of protein in the sample:

The peak in the gel filtration chromatography contained the following:

peak, ml	mODxml	mOD
5.5	64.61	11.74

If it is assumed that the specific ext is 1mg/OD, we will get:

Approx. mg prot. in 5.5ml 0.01174 meaning.approx 0.012 mg prot. frac.

Molar
concentration:

0.012 mg/(34000 mg/mM)

3.53E-07 mM = 352.9 pico mol

From the above calculations and assumptions, obtaining an amino acid sequence should be possible. The 5 mls of sample was desalted using the presorb system and 5 volumes of 20 mM Acetic acid. As indicated by the SDS PAGE, the sequencing results showed two proteins in the sample. Sequence A was of 57 amino acids from the N-terminal and the concentration of the protein was estimated to 130 p moles (Table12). Sequence B was of 38 amino acids and the concentration of the protein was estimated to 90 p moles (Table 13). According to SDS-PAGE this ratio was expected to be higher.

Table 12. SEQ. ID No. 1 obtained from purified inhibitor fraction.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
L	A	V	V	A	R	A	V	K	D	V	A	P	F	G	V	X	Y	D	T	K	T	L	G	N
26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
N	L	G	G	Y	A	V	P	N	Q	L	G	L	L	D	G	G	X	D	W	T	M	I	X	K
51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
N	S	M	V	D	V	K																		

Table 13. SEQ. ID No. 2 obtained from purified inhibitor fraction.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
G	P	P	L	A	P	V	T	E	A	P	A	T	S	L	Y	T	I	P	F	H	H	G	A	A
26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
X	V	L	D	V	X	S	S	X	L	L	W	X												

A data base search for homology to the sequenced poly-peptides came out negative. Neither of the poly-peptides have been sequenced or described before. A Swiss prot analysis of the A sequence shows a poly-peptide of 6.05KDa. The poly-peptide has 5 positively and 5 negatively charged amino acids and a theoretical pI of 6.14. A Kyte and Doolittle analysis of the poly-peptide shows no significant hydrophobicity nor hydrophilicity of the poly-peptide. According to instability index the poly-peptide is classified as stable.

Analysis of protease activity

Based on the above characterisations of the xylanase inhibitor. It can not be ruled out that the decrease in xylanase activity, when mixed with the flour extract, is not due to a proteolytic hydrolysis of the xylanase. Therefore, a purified xylanase was incubated with an "inhibitor" extract. As can be seen from Figure 10 and 11, no hydrolysis seems to occur.

As can be seen from the two chromatograms, no hydrolysis of the xylanase seems to occur. There is a little more back-ground in the chromatogram with active inhibitor (Figure 11). However, this back-ground corresponds to the chromatogram of the inhibitor alone (chromatogram of inhibitor not shown). The difference in background must be due to precipitation in the boiled inhibitor sample.

Effect of inhibitor on different xylanases

Several trials have been carried out to study the inhibition of different xylanases. First we believed that the decrease in xylanase activity was due to a proteolytic activity in the extract. Therefore, different xylanases were incubated with different volumes of "inhibitor" extract (Figure 12). The xylanases were found to be inhibited to different extends. What we also found was that there seemed to be an increase in inhibition as a function of "inhibitor" concentration.

The results illustrated in Figure 12 could indicate that the decrease was due to proteolysis or inhibitor. However, time course experiments with constant xylanase and inhibitor concentrations did not show decreased activity as a function of time. To be able to distinguish between protease and inhibitor, real kinetics has to be made (see "inhibitor kinetics").

Two *Bacillus subtilis* xylanases have been studied very closely regarding their baking performance. These xylanases differed a little in their functionality, meaning that one gave a slightly higher specific volume when baked in identical doses. One explanation could be different inhibition of their activity in the flour. An experiment was therefore performed to examine this. The experiment has been repeated twice, using two different kinds of flour as source for the inhibitor (Table 14).

Table 14. Inhibition of two xylanases (980601 and 980603) by inhibitor extracted from two kinds of flour (98002 and 98026). Inhibition is calculated as % inhibition and as % residual activity, compared to blank.

Flour			98002	98026	
Inhibation, %		980601	67.03	75.04	
		980603	60.76	61.33	
			98002	98026	Avg
Rest act., %		980601	32.97	24.96	28.96
		980603	39.24	38.67	38.96
Difference					25.65

The trial shows that the two xylanases are inhibited to different extents by the inhibitor. The xylanases differ in only six amino acids.

Based on 980601, three xylanase mutants have been made (XM1, XM2 and XM3). These mutants have been analysed for inhibition (Figure 13).

As can be seen from Figure 13 the three mutants differ in rest activity, meaning that they are inhibited to different degrees by the xylanase inhibitor. Four (BX, Röhm, XM1 and XM3) of the five xylanases have the same specific activity (approx. 25000 TXU/mg protein). XM2 is expected to have the same specific activity.

The difference in inhibition between XM1 and XM2 is approx. 250% (the residual activity of XM1 is 2.5 times higher than the rest activity of XM2). This difference is due to one amino acid. Amino acid 122 in XM2 is changed from arginine to asparagine, introducing less positive charge near the active site.

Inhibitor kinetics

Simple preliminary kinetics were performed. Just to be able to determine whether the inhibitor is competitive or non-competitive.

Different amounts of substrate were incubated with a constant xylanase- and inhibitor concentration (fig. 14).

As can be seen from figure 14, V_{max} for both xylanase with and without inhibitor is approx. 1.19. This indicates that the inhibition is competitive.

Inhibition as a function of pH

A simple spot for xylanase inhibitor at a different pH revealed that there seemed to be an effect of pH on the inhibition of the xylanases. Therefore, an experiment was set up to examine this effect. As can be seen from Figure 15 the inhibition of the xylanases are influenced by pH. Figure 16 illustrates the pH optima for the xylanases. If these two curves are compared, we see the highest inhibition at the pH optimum for the xylanase, except for the pH 4 measurement of the Novo xylanase (980901).

To determine whether the inhibition ratios measured in the assays reported here are relevant in the dough, some calculations can be made:

Inhibitor extraction

Gram flour:	6
ml water:	12
g flour/ml:	0.5
g flour in assay:	0.05

Xylanase solution

TXU/ml:	12
TXU/ml in assay:	3

Inhibitor : xylanase ratios

TXU/kg flour:	60000	in inhibitor assay
TXU/kg flour:	3000	in bakery applications

From the above calculations, the inhibitor: xylanase ratio in the assay can be calculated to be 20 times lower in the assay than in dough. This can only mean that the xylanase must be much more inhibited in dough. The mobility and water activity is much lower in dough and this might influence the inhibition.

Example 3

Baking trials.

The data below are from a baking trail with the XM1 mutant. The data show that this novel xylanase mutant is clearly superior to BX (*Bacillus subtilis* wild type) based on volume. Based on stickiness measurement there are no significant diffence between the two xylanases

Enzymes

980902 (BX): Purified *Bacillus sub.* wild type xylanase expressed in *E. coli.* (2000 TXU/ml)

980903 (XM1): Purified mutant of *Bacillus sub.* wild type xylanase expressed in *E. coli.* (1375 TXU/ml)

Flour

Danish flour, batch 98022.

Baking test (hard crust rolls)

Flour 2000 g, dry yeast 40 g, sugar 32 g, salt 32 g, GRINDSTED™ Panodan A2020 4 g, water 400 Brabender Units + 4% were kneaded in a Hobart mixer with hook for 2 minutes low speed and 9 minutes high speed. The dough temperature was 26°C. The dough was scaled to 1350 gram. Resting 10 minutes at 30°C followed by moulding on a Fortuna moulder. Proofing 45 minutes at 34°C, 85 % RH. Baked in a Bago-oven 18 minutes 220°C and steamed 12 seconds.

After cooling the rolls were scaled and their volume measured by the rape seed deplacement method.

$$\text{Specific volume} = \frac{\text{volume of the bread, ml}}{\text{weight of the bread, g}}$$

Stickiness measurement

Stickiness measurement was performed according to protocol 2.

As can be seen from Table 15 the novel xylanase mutant (XM1) gives rise to significant higher bread volume increase than BX.

Table 15. Bread volume increase (ml/gram) and stickiness (g x s) as function of two xylanases (BX and XM1) applied at different dosages.

Sample	Dose, TXU/kg	Stickiness, g x s	Specific vol., ml/g	Spec. vol. increase, %
BX	2000	6.00	6.03	2.55
BX	5000	6.60	6.49	10.37
BX	8000	5.00	6.77	15.14
BX	12000	7.00	6.72	14.29
XM1	2000	4.30	6.60	12.24
XM1	5000	6.20	6.88	17.01
XM1	8000	6.20	7.06	20.07
XM1	12000	6.90	7.32	24.49
Control	0	4.50	5.88	-

The data are shown in Figure 17, 18 and 19.

Example 4

Dough stickiness as a function of XM1, Röhm's Veron special xylanase and a purified version of Röhm's Veron special xylanase.

To determine whether the novel xylanase, XM1 gives more or less sticky dough than Röhm's Veron Special xylanase (and a purified version herof) dough were prepared and stickiness as function of xylanase was determined.

Flour

Danish flour, batch 98022 was used.

Dough preparation

Dough were prepared as described in protocol 2. After mixing the dough rested for 10 and 45 minutes, respectively, in sealed containers before stickiness measurement.

Stickiness measurement

Stickiness measurements were performed according to Protocol 2.

Enzymes

980903 (XM1): Purified mutant of *Bacillus sub.* wild type xylanase expressed in *E. coli.* (1375 TXU/ml)

#2199: Röhm's Veron Special xylanase (10500 TXU/g)

980603 (Röhm): Purified preparation of Frimond's Belase xylanase (identical to Röhm's) (1050 TXU/ml)

The following dough were made (Table 16)

Table 16. Dough made for determination of stickiness

Xylanase	Dosage, TXU/kg flour
980603 (Purified Röhm xylanase)	15.000
Control	0
XM1	15.000
#2199 (Röhm's Veron Special)	15.000

The dough in Table 16 gave the stickiness results in Table 17.

Table 17. Results from stickiness measurements on dough prepared with Purified Röhm xylanase, control, XM1 and Röhm's Veron Special xylanase.

Xylanase	TXU/ kg flour	Leavening time, min.	Stickiness, g x s	Stickiness increase, g x s
980603	15.000	10	7.22	2.22
980603	15.000	45	10.15	4.08
Control	0	10	5.00	0
Control	0	45	6.09	0
XM1	15.000	10	6.61	1.61
XM1	15.000	45	9.64	3.55
#2199	15.000	10	8.57	3.57
#2199	15.000	45	12.14	6.05

The data are shown in Figure 20, 21 and 22.

The increase in stickiness using the XM1 is lower than the stickiness increase with the purified Röhm xylanase. The stickiness increase obtained using the unpurified Röhm xylanase is much higher.

Example 5

Dough stickiness as a function of bacterial Endo-β-1,4-Glucanase

The results in the following is from an experiment designed to study the ability of bacteial Endo-β-1,4-Glucanase to give stickiness.

Enzymes

981102-1 (Xyl): Correspond to a purified preparation of Röhm's bacterial xylanase from the product Veron Special. The preparation is pure xylanase and do not contain any Endo-β-1,4-Glucanase (350 TXU/ml)

981102-2 (Xyl + Gluc): Correspond to a purified preparation of Röhm's bacterial xylanase from the product Veron Special, containing Endo-β-1,4-Glucanase (900 TXU/ml + 19 BGU/ml)

Xylanase Assay

Xylanase assays were performed according to Protocol 1

Glucanase assay

Glucanase assays were performed according to Protocol 4

Flour

Danish flour, batch no 98058 was used. The water absorbtions, at 400 BU is 60%.

Dough preparation

Dough were prepared as described in protocol 2. After mixing the dough rested for 10 and 45 minutes respectively at 30°C in sealed containers.

Stickiness measurement

Stickiness measurements were performed according to Protocol 2

The dough listed in Table 18 were prepared and examined for stickiness.

Table 18. Dough prepared for examining stickiness

Dough No.	Dough	TXU/kg flour	BGU/kg flour
1	Control	0	0
2	TXU	7500	0
3	TXU + BGU	7500	158
4	TXU	15000	0
5	TXU + BGU	15000	316

The dough listed in Table 18 gave the stickiness results in Table 19.

Table 19. Stickiness results from dough with xylanase and xylanase + glucanase. Dough No. refers to the dough No. in Table 18. Stik_10 indicate results from stickiness measurements after 10 minutes and Stik_45 indicate measurements after 45 minutes of resting.

Dough No.	Stik_10, g x s	std.dev	Stik_45, g x s	std.dev
1	4.5	0.342	5.11	0.552
2	5.29	0.619	8.62	0.607
3	5.47	0.663	9.38	0.832
4	8.61	0.408	9.15	0.418
5	8.73	0.35	10.19	0.857

As can be seen from Table 19. The Endo-β-1,4-Glucanase addition to the dough increases the stickiness of the dough.

The results from Table 19 are illustrated in Figure 23.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in biochemistry and biotechnology or related fields are intended to be within the scope of the following claims.

SEQUENCE LISTINGS

The following pages present a series of sequence listings - which include the following sequences:

SEQ ID NO. 1 - amino acid sequence from the inhibitor

SEQ ID NO. 2 - amino acid sequence from the inhibitor

SEQ ID NO. 3 - amino acid sequence of a wild type xylanase (R)

SEQ ID NO. 4 - nucleotide sequence of a wild type xylanase (R)

SEQ ID NO. 5 - amino acid sequence of a wild type xylanase (D)

SEQ ID NO. 6 - nucleotide sequence of a wild type xylanase (D)

SEQ ID NO. 7 - amino acid sequence of a mutant xylanase (XM1)

SEQ ID NO. 8 - nucleotide sequence of a mutant xylanase (XM1)

SEQ ID NO. 9 - amino acid sequence of a mutant xylanase (XM2)

SEQ ID NO. 10 - nucleotide sequence of a mutant xylanase (XM2)

SEQ ID NO. 11 - amino acid sequence of a mutant xylanase (XM3)

SEQ ID NO. 12 - nucleotide sequence of a mutant xylanase (XM3)

Notes:

For XM1 - we introduced changes (as shown) in the nucleotide sequence and amino acid sequence by site directed mutation of the gene. The mutated gene may be expressed in *E. coli*, in Bacillus or in any organism of choice.

SEQ ID NO. 1

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
L	A	V	V	A	R	A	V	K	D	V	A	P	F	G	V	X	Y	D	T	K	T	L	G	N
26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
N	L	G	G	Y	A	V	P	N	Q	L	G	L	L	D	G	G	X	D	W	T	M	I	X	K
51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
N	S	M	V	D	V	K																		

SEQ ID NO. 2

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
G	P	P	L	A	P	V	T	E	A	P	A	T	S	L	Y	T	I	P	F	H	H	G	A	A
26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
X	V	L	D	V	X	S	S	X	L	L	W	X												

SEQ ID NO. 3

AA :

MFKFKKKFLV GLTAAFMSIS MFSATASAAG TDYWQNWTDG GGTVNAVNGS GGNYSVNWSN
TGNFVVGKGW TTGSPFRTIN YNAGVWAPNG NGYLTLYGWT RSPLIEYYVV DSWGTYRPTG
TYKGTVKSDG GTYDIYTTTR YNAPSIDGDN TTFTQYWSVR QSKRPTGSNA AITFSNHVNA
WKSHGMNLGS NWAYQVLATE GYKSSGSSNV TVW

SEQ ID NO. 4

DNA :

```
   1  ATGTTTAAGT TTAAAAAGAA ATTCTTAGTT GGATTAACGG CAGCTTTCAT GAGTATCAGC
  61  ATGTTTTCGG CAACCGCCTC TGCAGCTGGC ACAGATTACT GGCAAAATTG GACTGACGGG
 121  GGCGGGACAG TAAACGCAGT CAATGGCTCT GGCGGAAATT ACAGTGTTAA TTGGTCTAAT
 181  ACCGGGAATT TCGTTGTTGG TAAAGGCTGG ACTACAGGCT CGCCATTTAG AACAATAAAC
 241  TATAATGCCG GTGTTTGGGC GCCGAATGGC AATGGATATT TAACTTTATA TGGCTGGACG
 301  AGATCGCCCC TCATCGAATA TTATGTGGTG GATTCATGGG GTACTTACAG ACCTACCGGA
 361  ACGTATAAAG GTACCGTAAA GAGTGATGGA GGTACATATG ACATATATAC AACGACACGT
 421  TATAACGCAC CTTCCATTGA TGGCGATAAC ACTACTTTTA CGCAGTACTG GAGTGTCCGC
 481  CAGTCGAAGA GACCGACCGG AAGCAACGCT GCAATCACTT TCAGCAATCA TGTTAACGCA
 541  TGGAAGAGCC ATGGAATGAA TCTGGGCAGT AATTGGGCTT ATCAAGTCTT AGCGACAGAA
 601  GGATATAAAA GCAGCGGAAG TTCTAATGTA ACAGTGTGGT AA
```

SEQ ID NO. 5

Bacillus subtilis wild type xylanase :

AA :

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TGNFVVGKGW TTGSPFRTIN YNAGVWAPNG NGYLTLYGWT RSPLIEYYVV DSWGTYRPTG
TYKGTVKSDG GTYDIYTTTR YNAPSIDGDR TTFTQYWSVR QSKRPTGSNA TITFSNHVNA
WKSHGMNLGS NWAYQVMATE GYQSSGSSNV TVW

SEQ ID NO. 6

DNA :

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 121  GGCGGTATAG TAAACGCTGT CAATGGGTCT GGCGGGAATT ACAGTGTTAA TTGGTCTAAT
 181  ACCGGAAATT TTGTTGTTGG TAAAGGTTGG ACTACAGGTT CGCCATTTAG GACGATAAAC
 241  TATAATGCCG GAGTTTGGGC GCCGAATGGC AATGGATATT TAACTTTATA TGGTTGGACG
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 541  TGGAAGAGCC ATGGAATGAA TCTGGGCAGT AATTGGGCTT ACCAAGTCAT GGCGACAGAA
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```

Mutant XM1 :

SEQ ID NO. 7

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WKSHGMNLGS NWAYQVLATE GYKSSGSSNV TVW

SEQ ID NO. 8

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 181  ACCGGAAATT TTGTTGTTGG TAAAGGTTGG ACTACAGGTT CGCCATTTAG GACGATAAAC
 241  TATAATGCCG GAGTTTGGGC GCCGAATGGC AATGGATATT TAACTTTATA TGGTTGGACG
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 541  TGGAAGAGCC ATGGAATGAA TCTGGGCAGT AATTGGGCTT ACCAAGTCCT CGCGACAGAA
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Mutant XM2 :

SEQ ID No. 9

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SEQ ID No. 10

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 481  CAGTCGAAGA GACCAACCGG AAGCAACGCT GCTATCACTT TCAGCAATCA TGTGAACGCA
 541  TGGAAGAGCC ATGGAATGAA TCTGGGCAGT AATTGGGCTT ACCAAGTCCT CGCGACAGAA
 601  GGATATAAAA GTTCCGGAAG TTCTAACGTA ACAGTGTGGT AA
```

Mutant XM3 :

SEQ ID NO. 11

AA :

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SEQ ID NO. 12

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 241  TATAATGCCG GAGTTTGGGC GCCGAATGGC AATGGATATT TAACTTTATA TGGTTGGACG
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 541  TGGAAGAGCC ATGGAATGAA TCTGGGCAGT AATTGGGCTT ACCAAGTCAT GGCGACAGAA
 601  GGATATCAAA GTAGTGGAAG TTCTAACGTA ACAGTGTGGT AA
```

CLAIMS

1. An endo-β-1,4-xylanase inhibitor obtainable from wheat flour.

2. An inhibitor according to claim 1, wherein the inhibitor has a MWT of about 34 kDa (as measured by gel filtration or SDS page).

3. An inhibitor according to claim 1 or claim 2, wherein the inhibitor has a pI of about 6.

4. An inhibitor according to any one of the preceding claims wherein the inhibitor comprises the amino acid sequence presented as SEQ. ID No. 1 and/or the amino acid sequence presented as SEQ. ID No. 2, or a variant, homologue, or fragment thereof.

5. An inhibitor according to any one of claims 1 to 4 in an isolated form.

6. A method for determining the degree of resistance of a xylanase to a xylanase inhibitor, wherein the method comprises:

(a) contacting a xylanase of interest with an inhibitor as defined in any one of the preceding claims; and

(b) determining the extent to which the inhibitor inhibits (if at all) the activity of the xylanase of interest.

7. A xylanase identified by a method according to claim 6, wherein the xylanase has a high degree of resistance to the inhibitor.

8. A foodstuff comprising a xylanase according to claim 7, preferably wherein the foodstuff is a bakery product.

9. A process comprising the steps of:

(a) performing a method according to claim 6;

(b) identifying one or more xylanases having a high degree of resistance to the inhibitor;

(c) preparing a quantity of those one or more identified xylanases.

10. A process comprising the steps of:

(a) performing a method according to claim 6;

(b) identifying one or more xylanases having a high degree of resistance to the inhibitor; and

(c) preparing a dough comprising the one or more identified xylanases.

11. A method for identifying a bacterial xylanase or mutant thereof suitable for use in the preparation of a baked foodstuff, the method comprising

(a) incorporating a bacterial xylanase of interest in a dough mixture; and

(b) determining the stickiness of the resultant dough mixture;

such that the bacterial xylanase or mutant thereof is suitable for use in the preparation of a baked foodstuff if the resultant dough mixture has a stickiness that is less than a similar dough mixture comprising a fungal xylanase.

12. A foodstuff comprising a suitable bacterial xylanase or mutant thereof identified by a method according to claim 11, preferably wherein the foodstuff is a bakery product.

13. A process comprising the steps of:

(a) performing a method according to claim 12;

(b) identifying one or more xylanases suitable for use in the preparation of a baked foodstuff;

(c) preparing a quantity of those one or more identified xylanases.

14. A process comprising the steps of:

(a) performing a method according to claim 12;

(b) identifying one or more xylanases suitable for use in the preparation of a baked foodstuff; and

(c) preparing a dough comprising the one or more identified xylanases.

15. Use of a bacterial xylanase or mutant thereof that is capable of being identified as being suitable by a method according to claim 12 for the preparation of a non-sticky dough.

16. A method for identifying a xylanase composition or a medium in which a xylanase is to be prepared or a medium to which a xylanase is to be added that is to be suitable for use in the preparation of a baked foodstuff, the method comprising

(a) providing a composition containing the xylanase of interest or a medium in which the xylanase is to be prepared or a medium to which the xylanase is to be added; and

(b) determining the presence of active glucanase enzyme(s) in the composition or medium;

such that if there is at most a low level of active glucanase enzyme(s) in the composition or medium then that composition or medium is suitable for the preparation of a baked foodstuff.

17. A foodstuff comprising a suitable composition or medium identified by a method according to claim 16, preferably wherein the foodstuff is a bakery product.

18. A process comprising the steps of:

(a) performing a method according to claim 16;

(b) identifying one or more compositions or mediums suitable for use in the preparation of a baked foodstuff;

(c) preparing a quantity of those one or more identified compositions or mediums.

19. A process comprising the steps of:

(a) performing a method according to claim 16;

(b) identifying one or more identified compositions or mediums suitable for use in the preparation of a baked foodstuff; and

(c) preparing a dough comprising the one or more identified identified compositions or mediums.

20. Use of a composition or medium that is capable of being identified as being suitable by a method according to claim 16 for the preparation of a non-sticky dough.

21. A method comprising:

(a) determining the amount of inhibitor according to any one of claims 1 to 4 in a wheat flour, which wheat flour may be wheat flour *per se* or may be present in a medium comprising same;

(b) selecting a suitable xylanase for addition to the wheat flour and/or selecting a suitable amount of a xylanase for addition to the wheat flour; and

(c) adding the suitable xylanase and/or suitable amount of the xylanase to the wheat flour.

22. A combination method comprising a first step comprising the method of claim 6 and a second step comprising the method of claim 11.

23. A combination method comprising two or more of the following steps: a first step comprising the method of claim 6, a second step comprising the method of claim 11, a third step comprising the method of claim 16; and a fourth step comprising the method of claim 21.

24. An amino acid sequence comprising any one of the amino acid sequences presented as SEQ ID No. 7, SEQ ID No. 9 or SEQ ID No. 11, or a variant, homologue or fragment thereof.

25. A nucleotide sequence encoding the amino acid sequence as defined in claim 23.

26. A nucleotide sequence selected from:

(a) a nucleotide sequence comprising any one of the nucleotide sequences presented as SEQ ID No. 8, SEQ ID No. 10 or SEQ ID No. 12, or a variant, homologue or fragment thereof;

(b) any one of the nucleotide sequences presented as SEQ ID No. 8, SEQ ID No. 10 or SEQ ID No. 12, or the complement thereof;

(c) a nucleotide sequence capable of hybridising any one of the nucleotide sequences presented as SEQ ID No. 8, SEQ ID No. 10 or SEQ ID No. 12, or a fragment thereof;

(d) a nucleotide sequence capable of hybridising to the complement any one of the nucleotide sequences presented as SEQ ID No. 8, SEQ ID No. 10 or SEQ ID No. 12, or a fragment thereof; and

(e) a nucleotide sequence which is degenerate as a result of the genetic code to the nucleotides defined in (a), (b), (c) or (d).

27. A nucleotide sequence according to claim 25 or claim 26 operably linked to a promoter.

28. A vector comprising the nucleotide sequence according to any one of claims 25 to 27.

29. A transformed host cell comprising the nucleotide sequence according to any one of claims 25 to 27.

30. A host cell comprising the nucleotide sequence according to any one of claims 25 to 27, wherein the nucleotide sequence is heterologous to the genome of the cell.

31. A process of preparing an enzyme according to 24 comprising expressing an appropriate nucleotide sequence according to any one of claims 25 to 27.

32. Use of an amino acid sequence presented as any one of SEQ ID No.s 7, 9, 11, or a variant, derivative or homologue thereof, to prepare a foodstuff.

33. A foodstuff comprising or prepared from an amino acid sequence presented as any one of SEQ ID No.s 7, 9, 11, or a variant, derivative or homologue thereof.

34. Use of an amino acid sequence presented as any one of SEQ ID No.s 3, 5, 7, 9, 11, or a variant, derivative or homologue thereof, to prepare a dough that is less sticky than a dough comprising a fungal xylanase.

35. A xylanase from *Bacillus subtilis* or a mutant thereof, wherein the xylanase is suitable for preparing non-sticky dough.

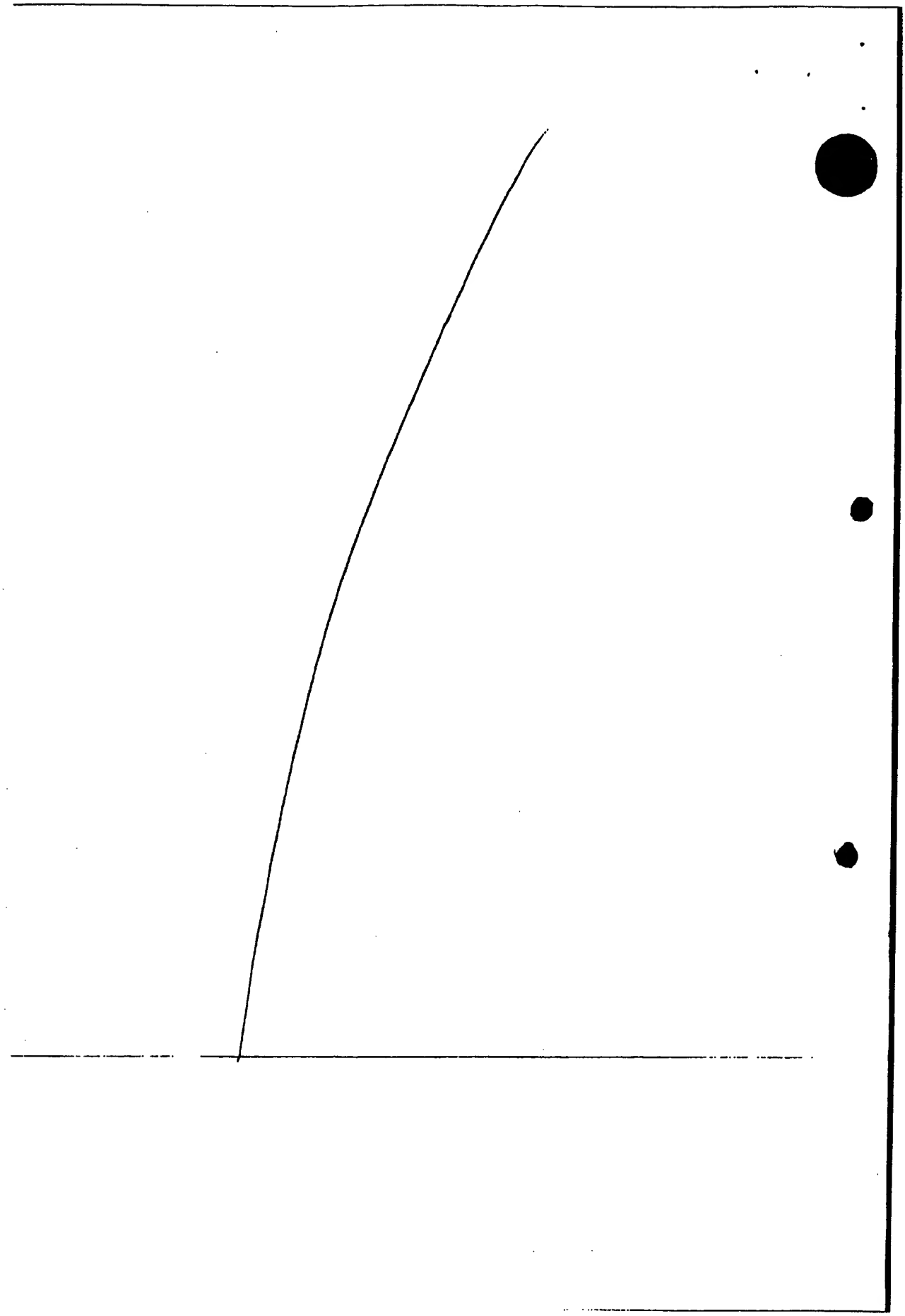
36. A *Bacillus subtilis* strain capable of producing a xylanase suitable for preparing non-sticky dough.

37. A bakery product prepared using the invention of any one of the preceding claims.

ABSTRACT

PROTEINS

The present invention discloses an endo-β-1,4-xylanase inhibitor as well as xylanases.



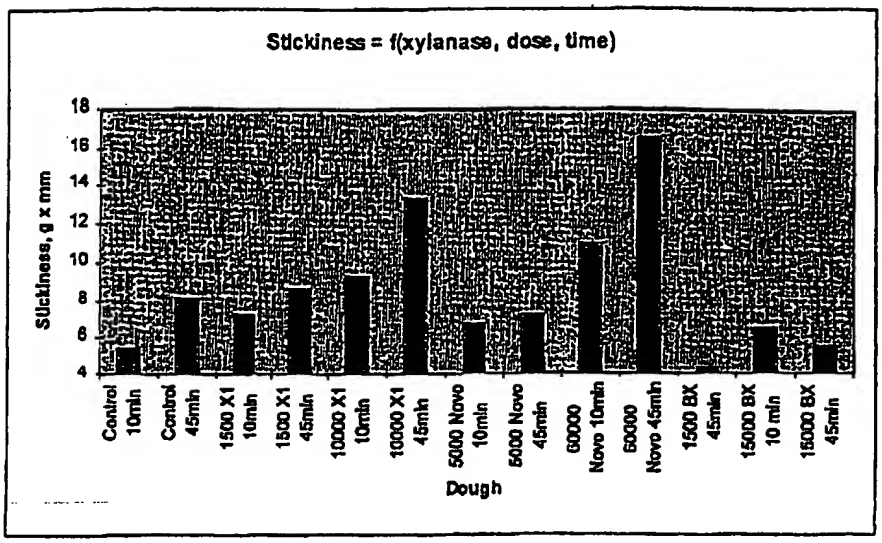
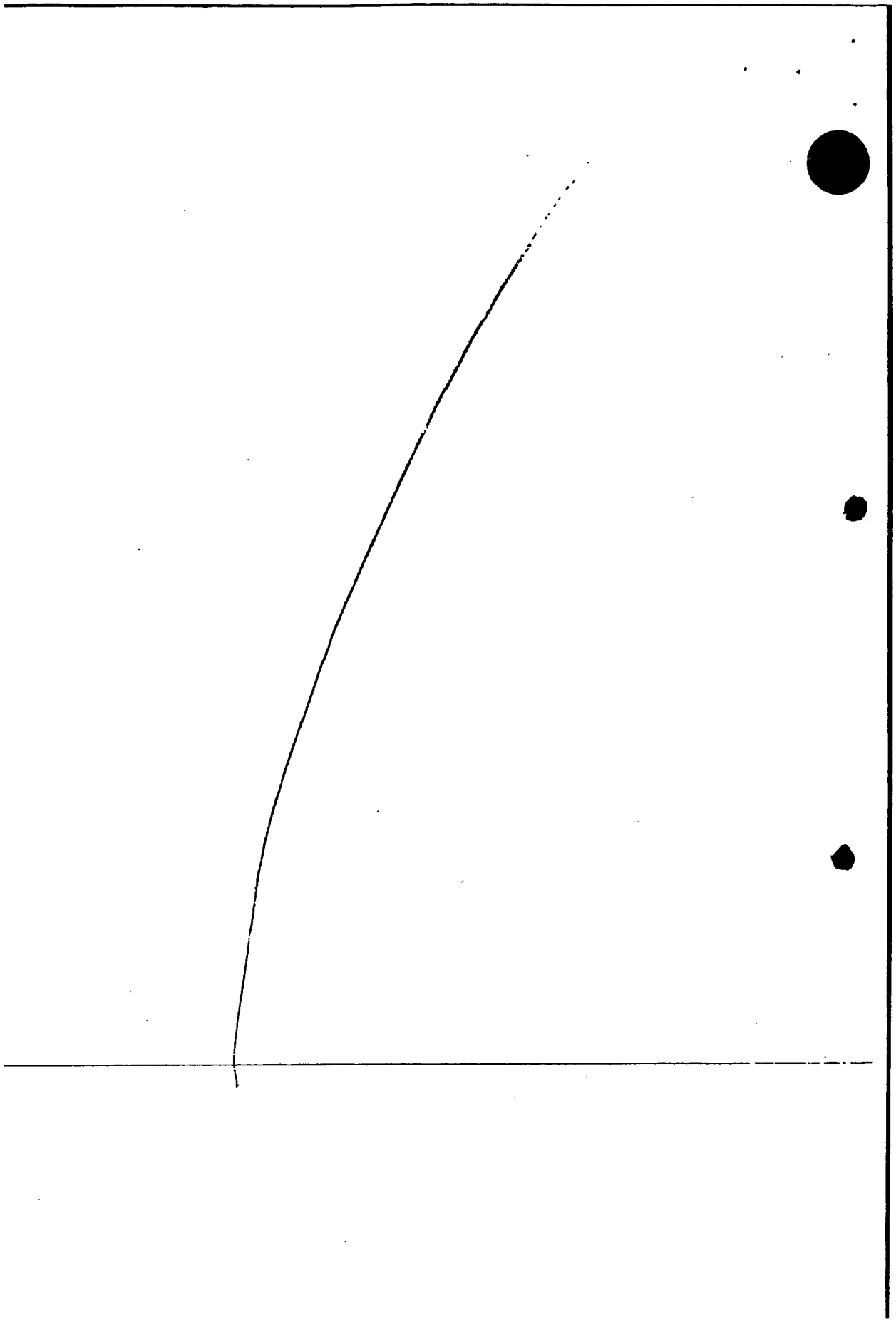


Fig. 1. Stickiness as a function of xylanases, dose and resting time.



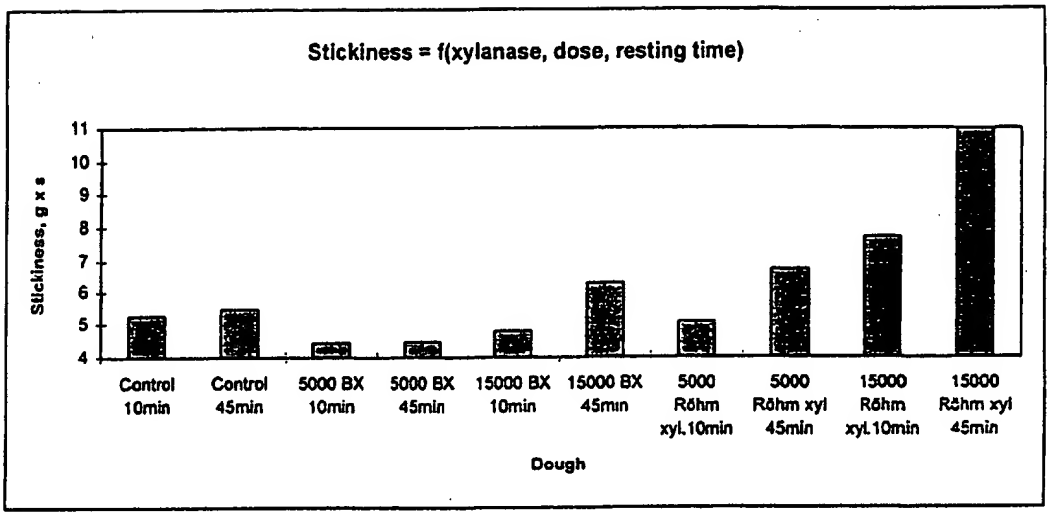
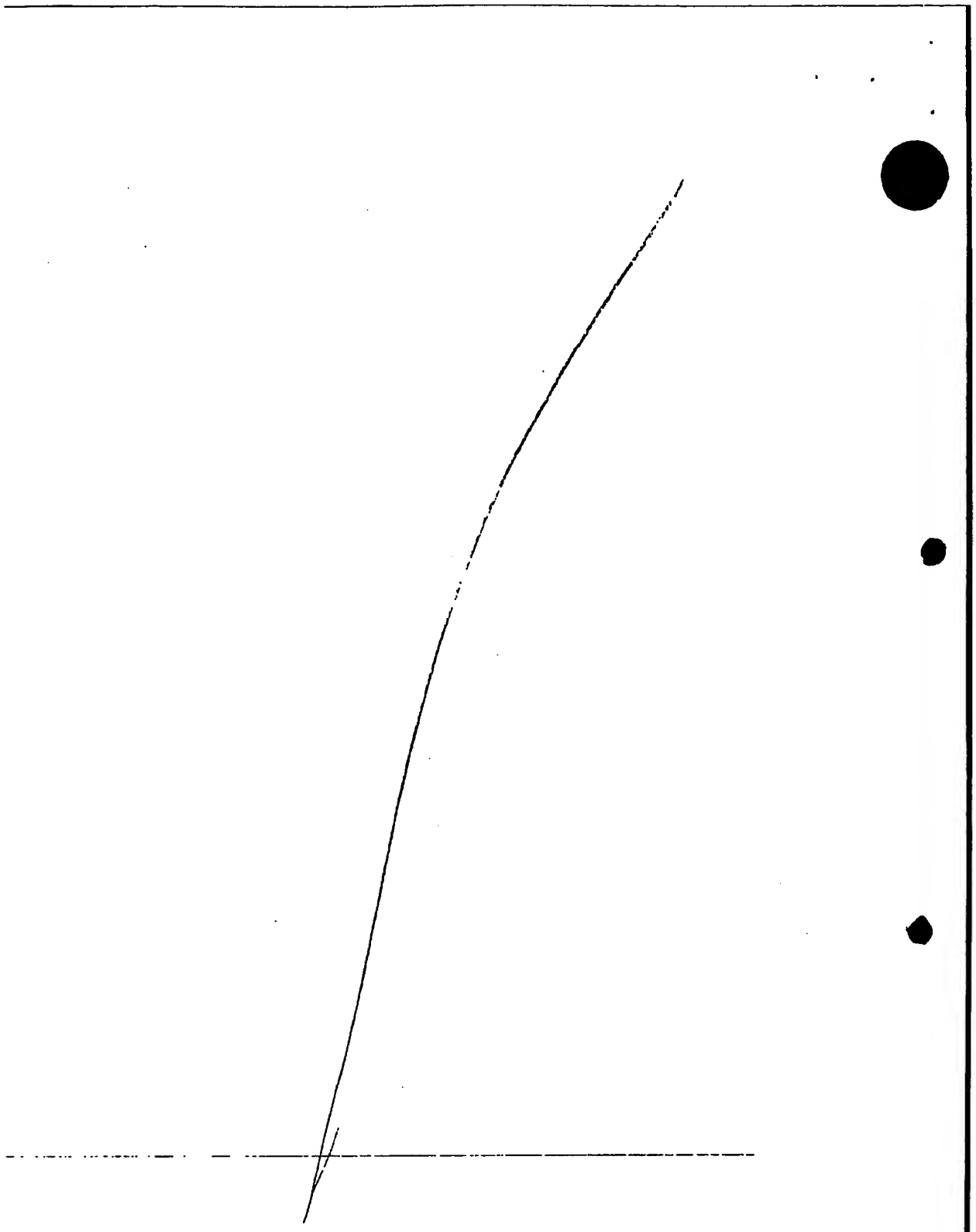

Stickiness = f(xylanase, dose, resting time)
Stickiness, g x s
11
10
9
8
7
6
5
4
Control 10min
Control 45min
5000 BX 10min
5000 BX 45min
15000 BX 10min
15000 BX 45min
5000 Röhm xyl.10min
5000 Röhm xyl 45min
15000 Röhm xyl.10min
15000 Röhm xyl 45min
Dough

Figure 2.



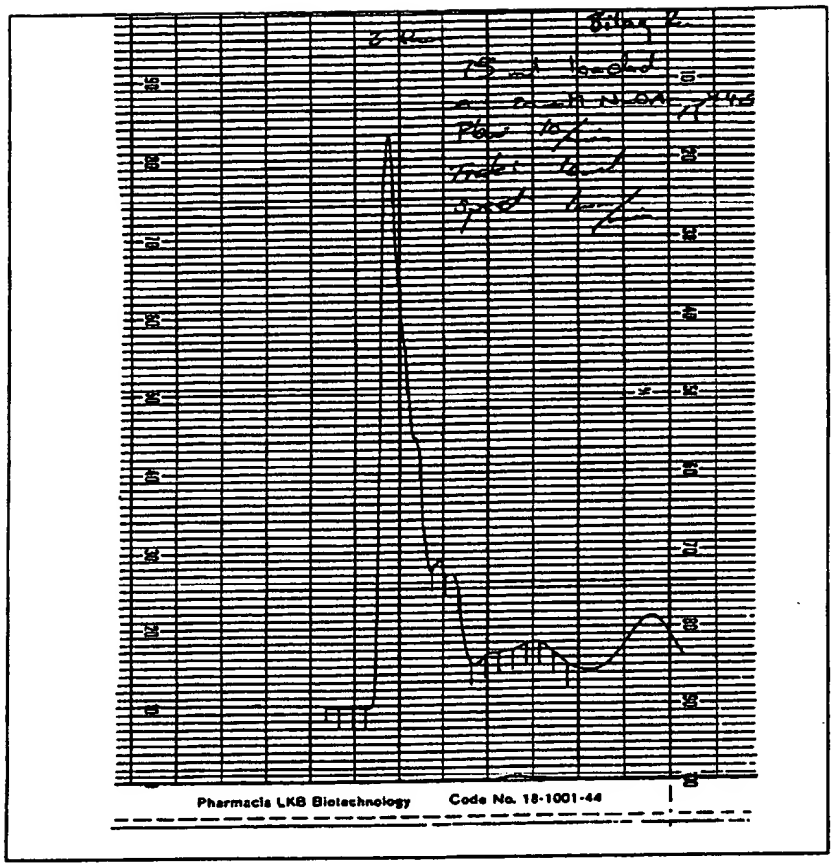
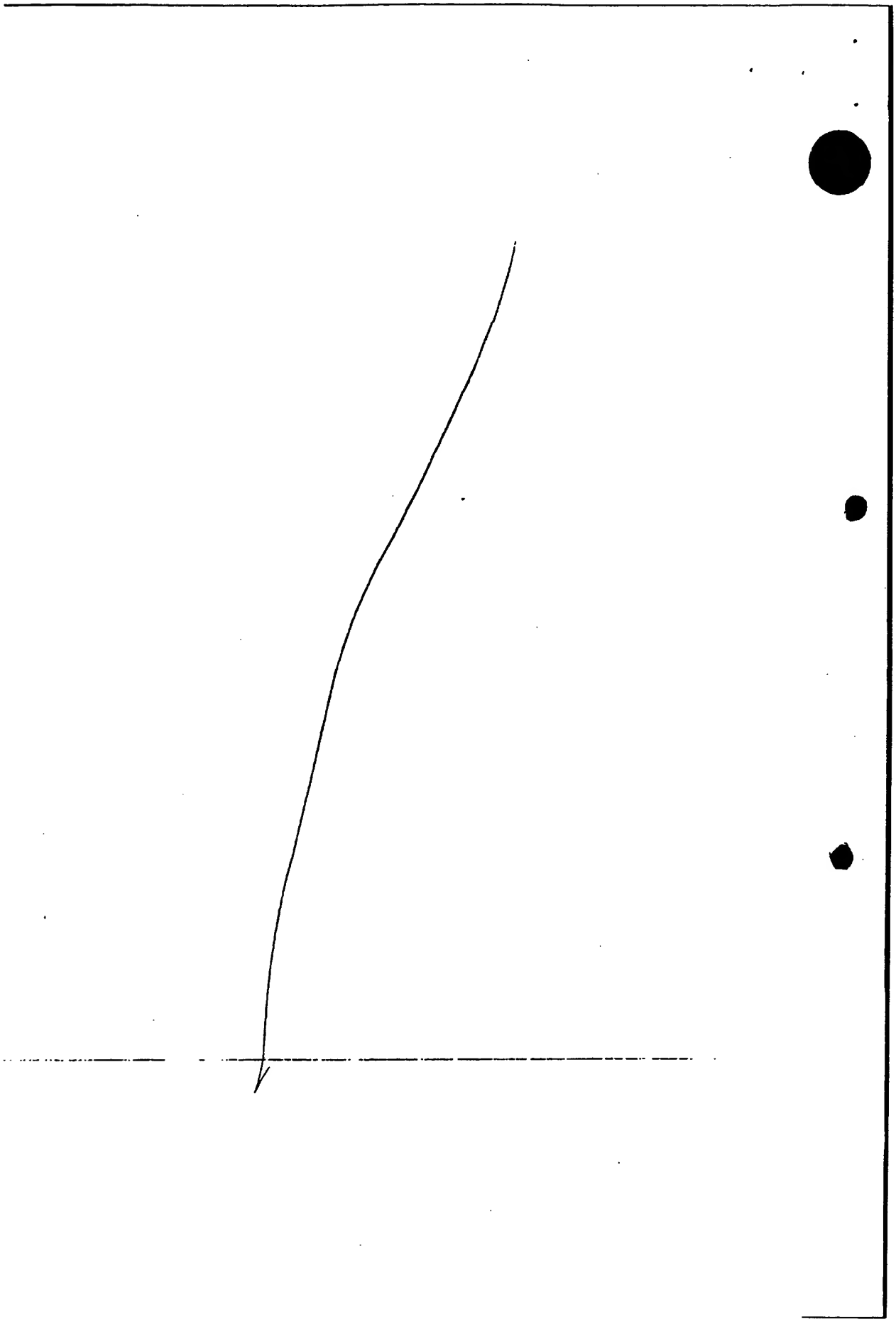


Fig. 3.



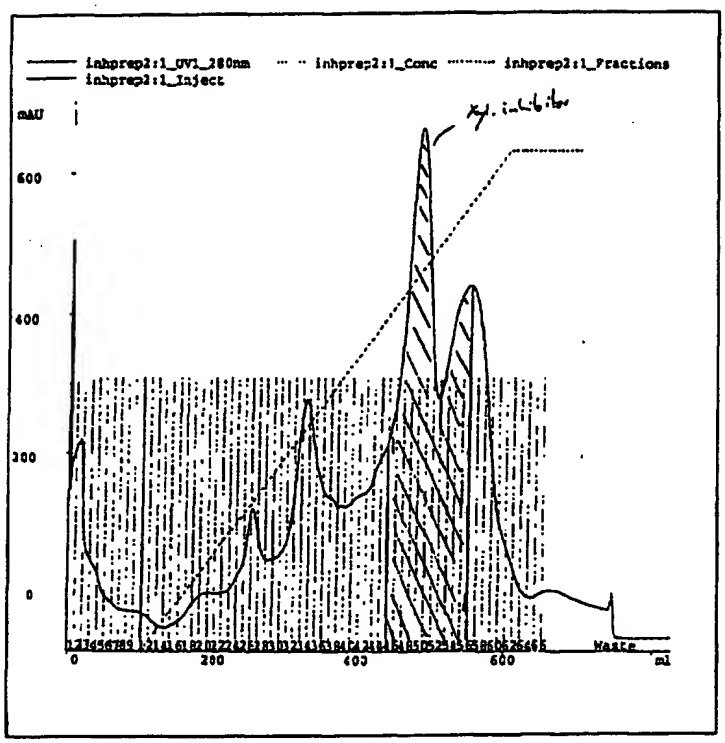

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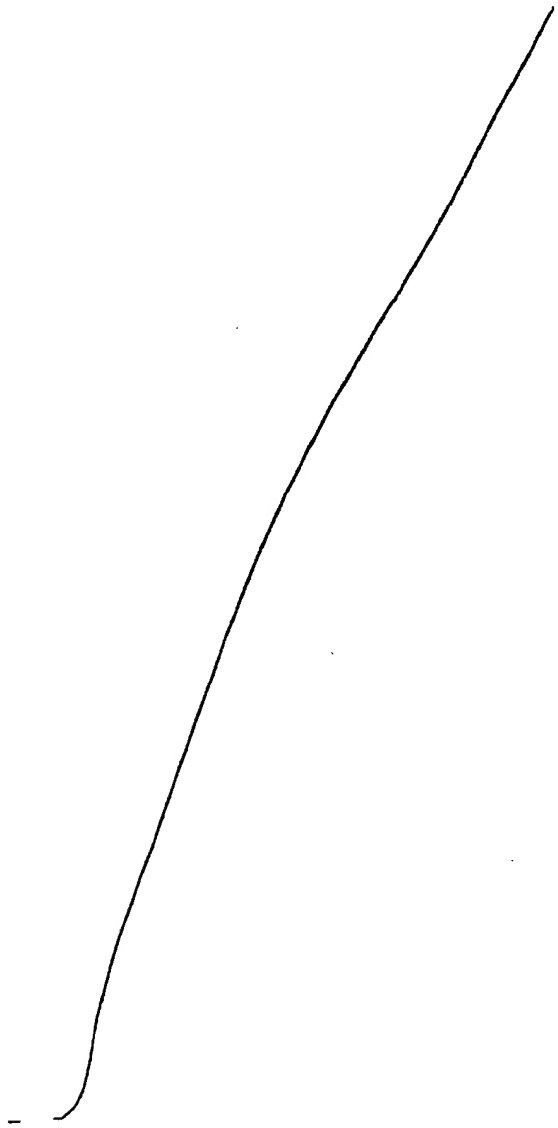
Figure 4.



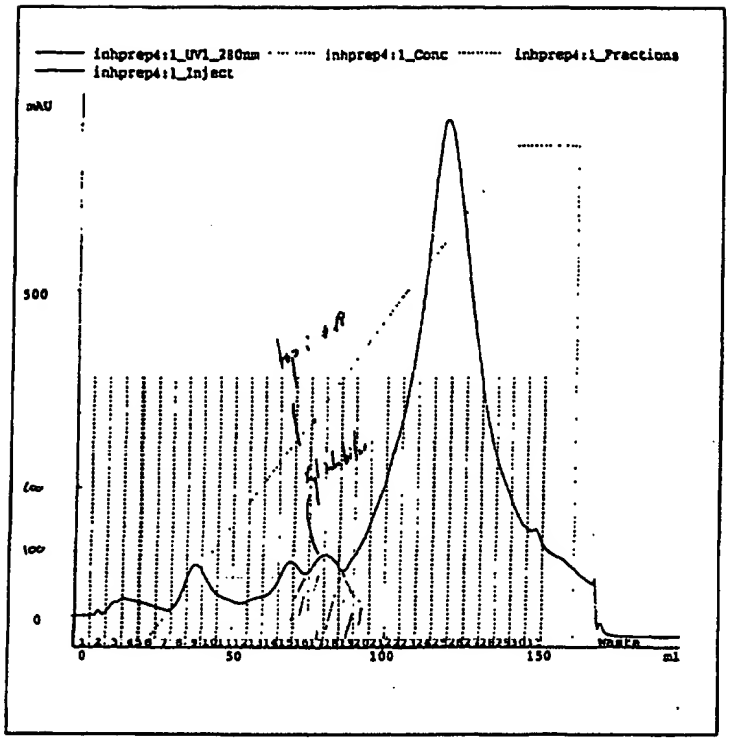

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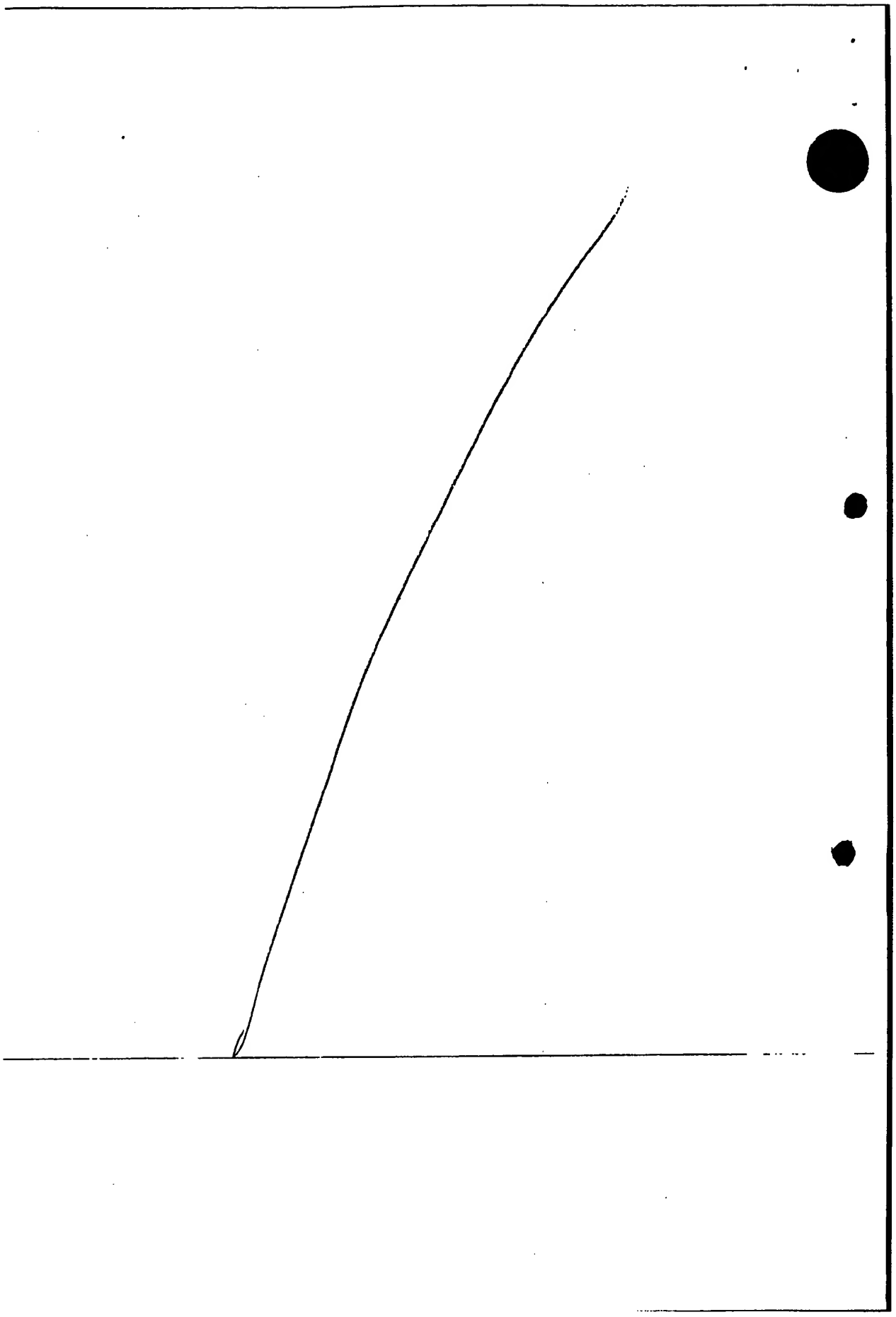
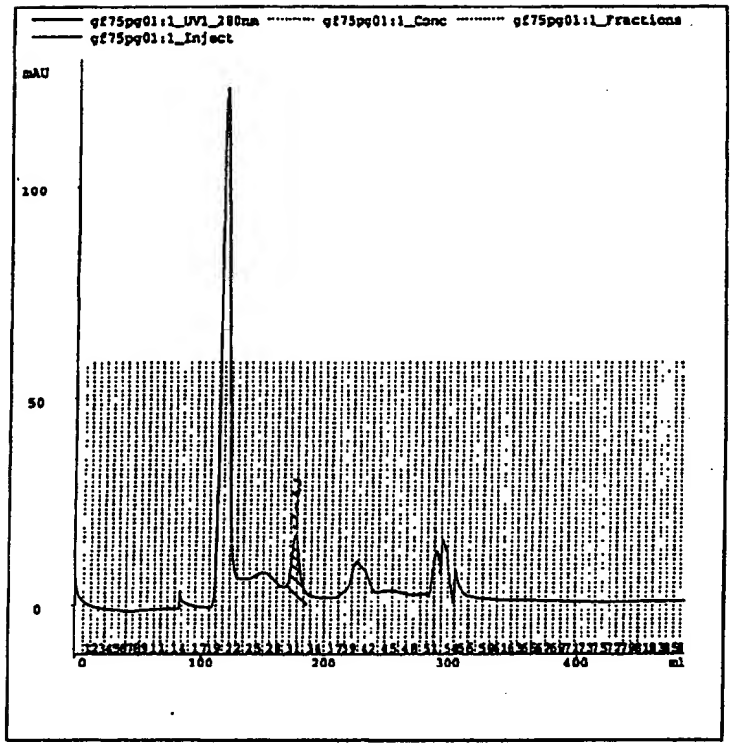
Figure 5.

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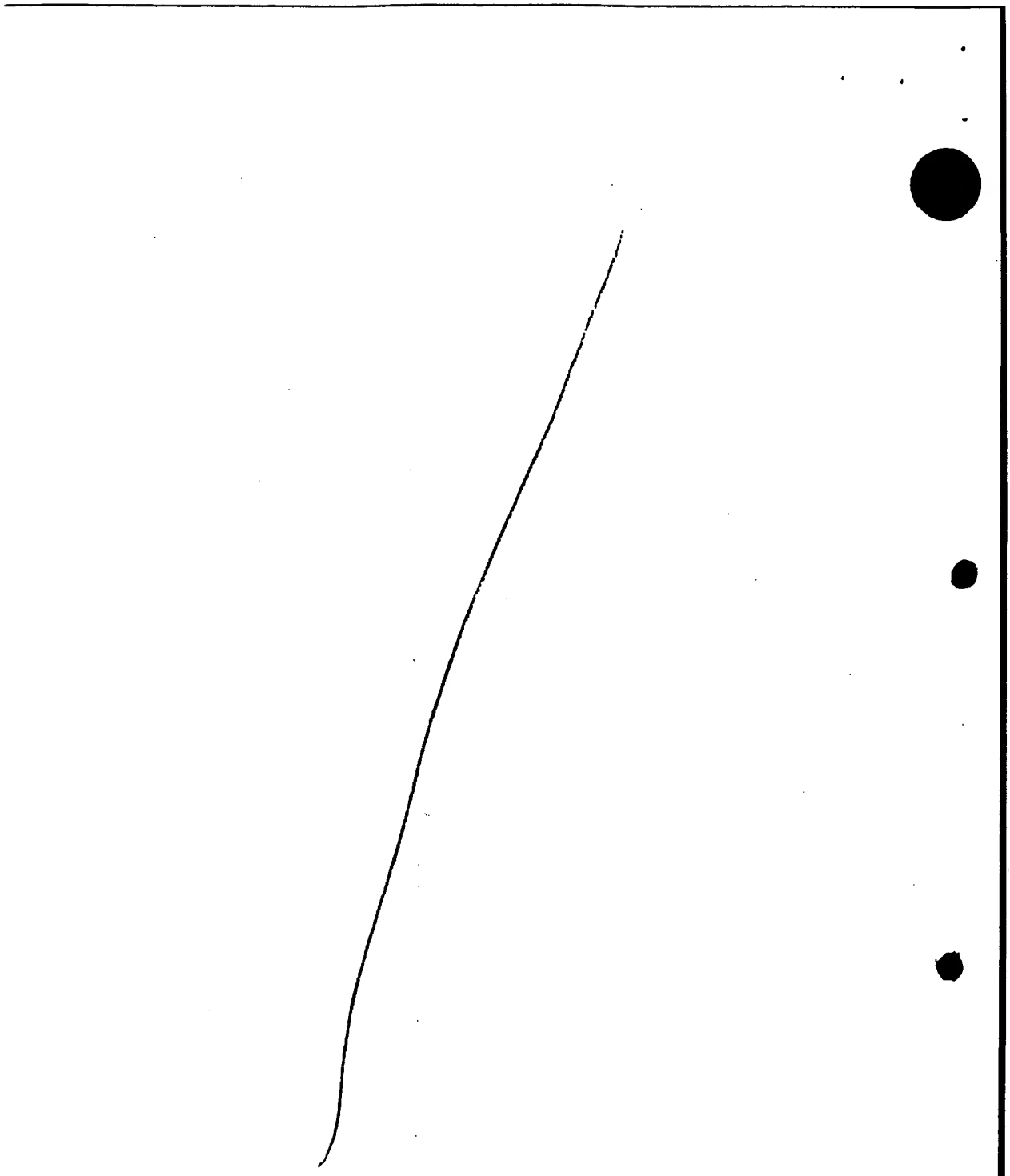

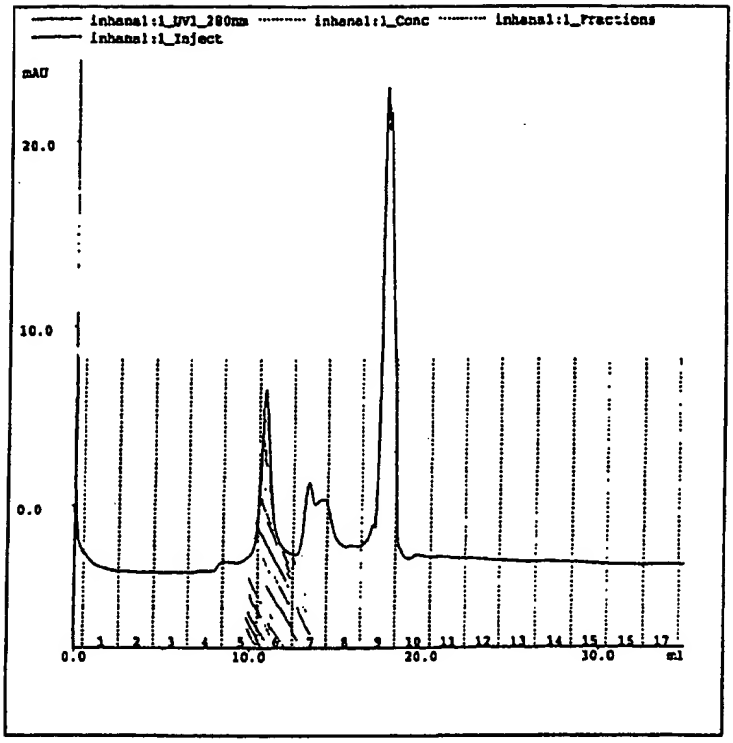
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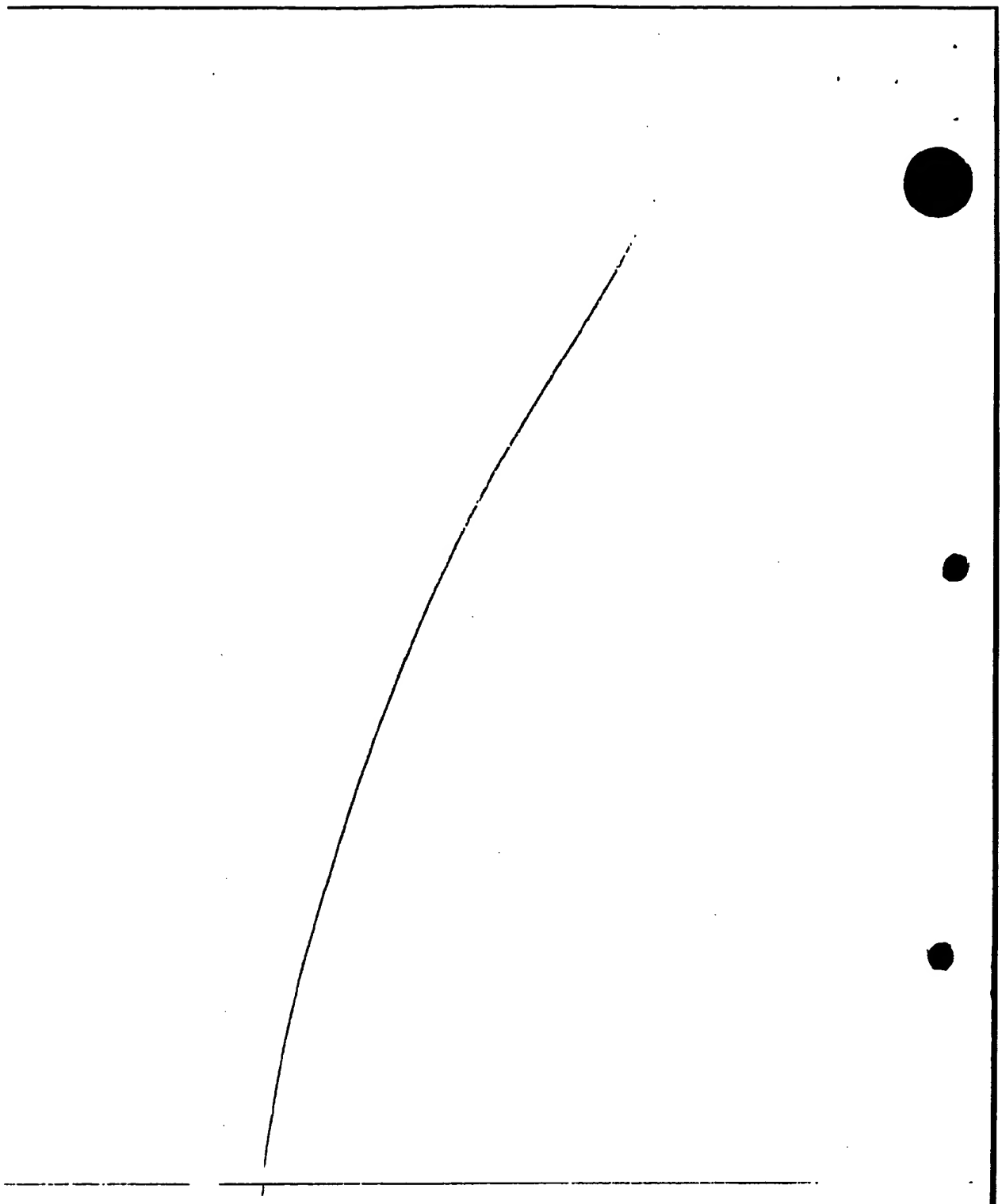
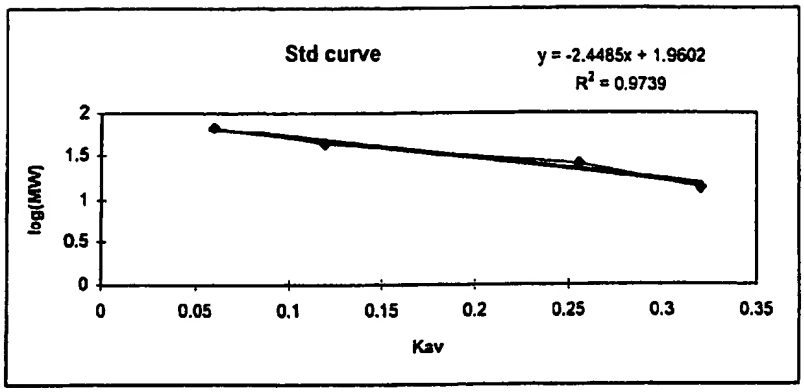
Figure 7.




Std curve
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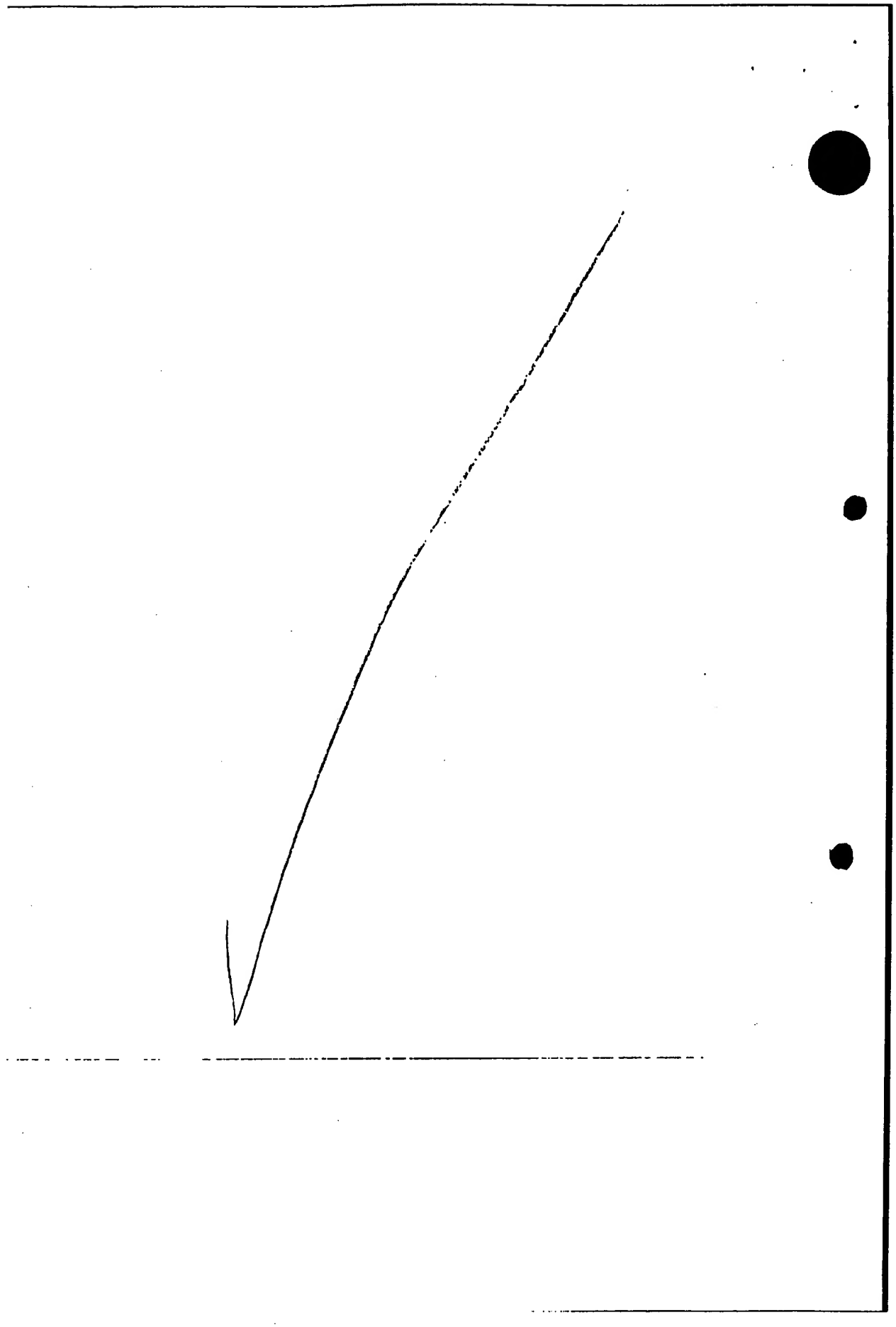
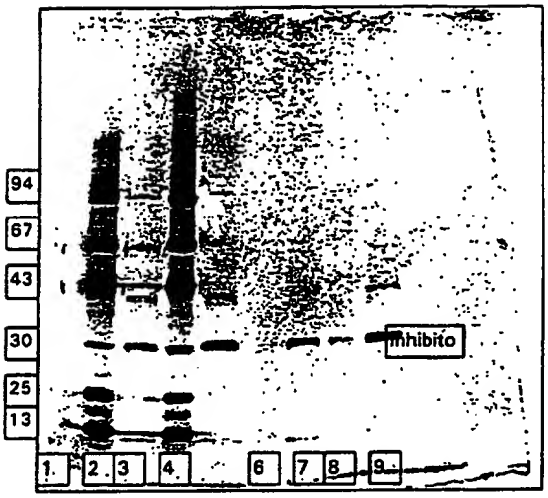
0.15
0.2
0.25
0.3
0.35
Kav

Figure 8.





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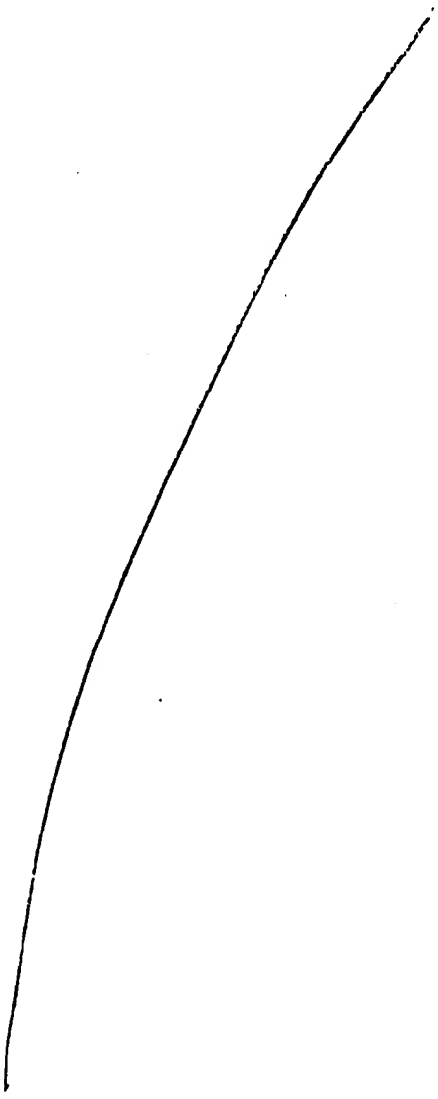

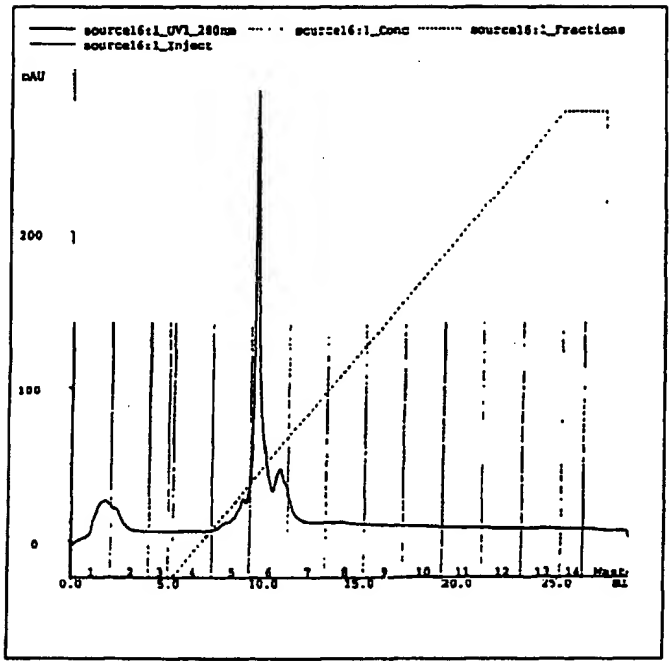
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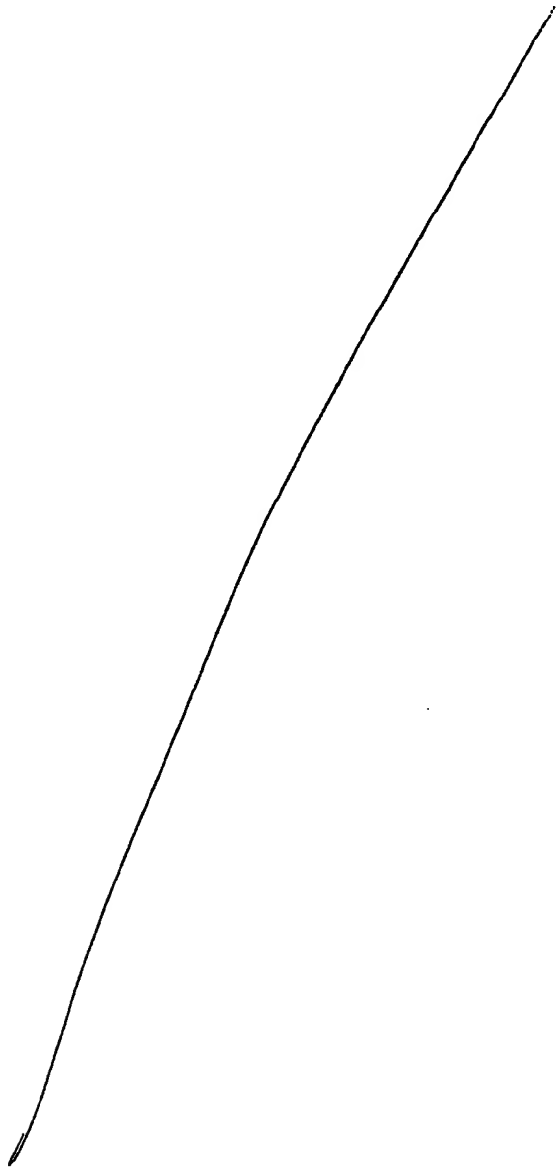
20.0
25.0
Min

Figure 10.



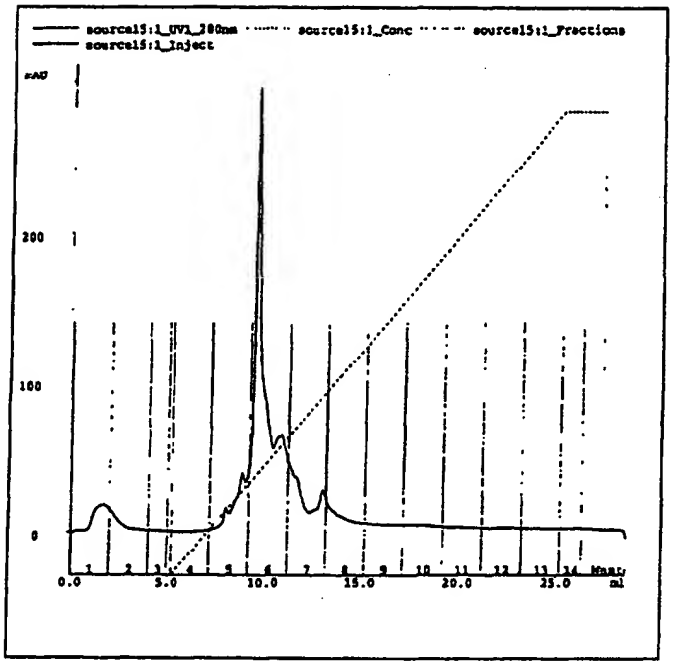
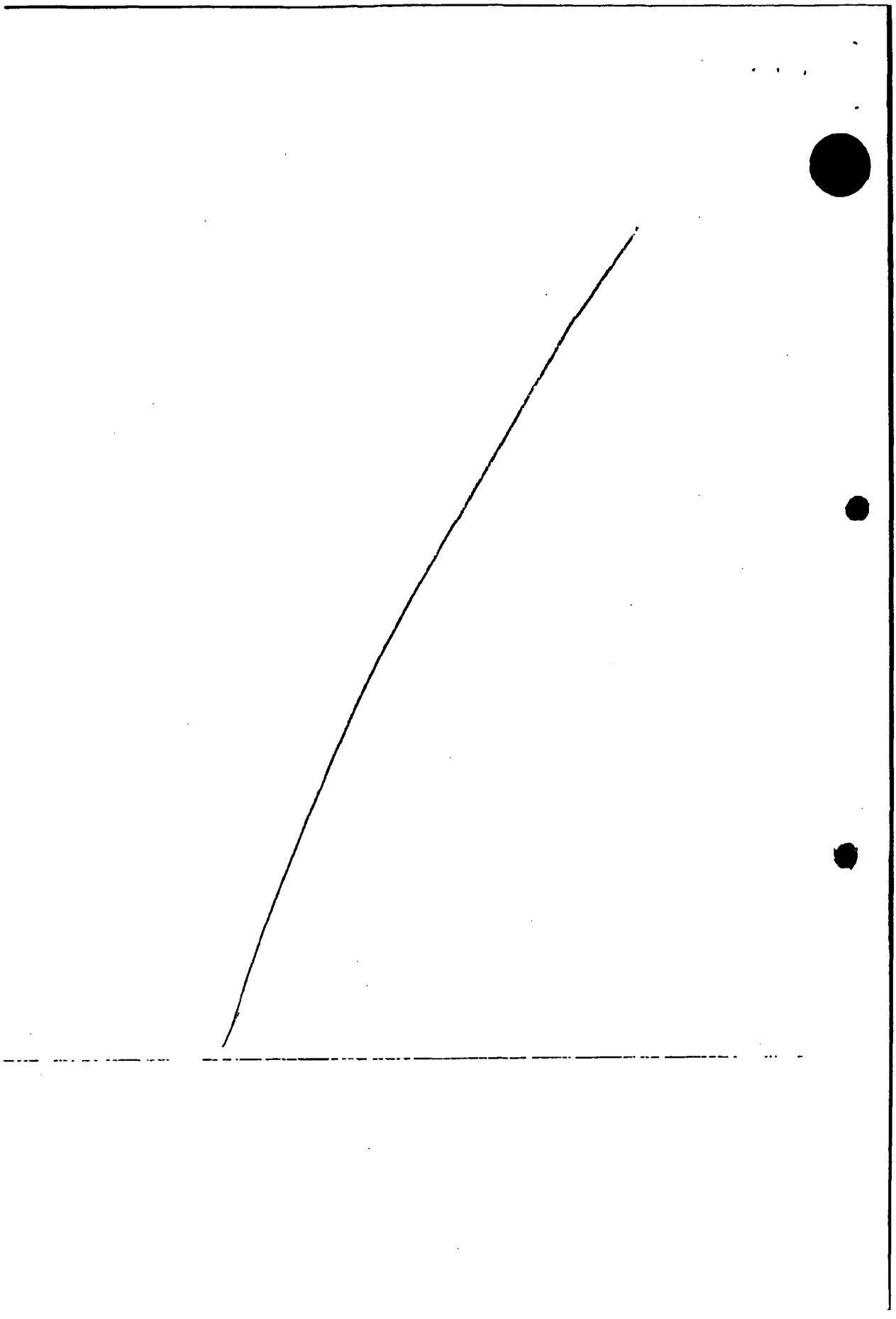

source15:1_UV1_280nm
source15:1_Conc
source15:1_Fractions
source15:1_Inject
mAU
200
100
0
0.0
5.0
10.0
15.0
20.0
25.0
Minutes
ml

Figure 11.



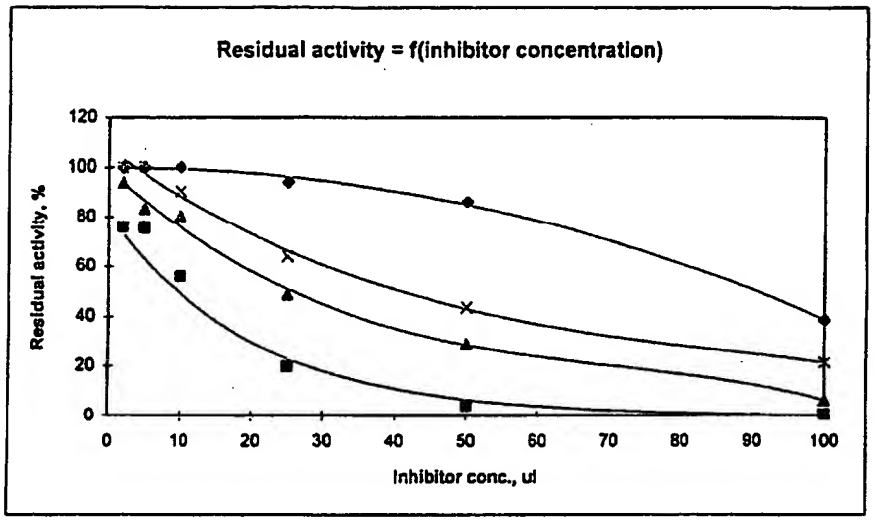
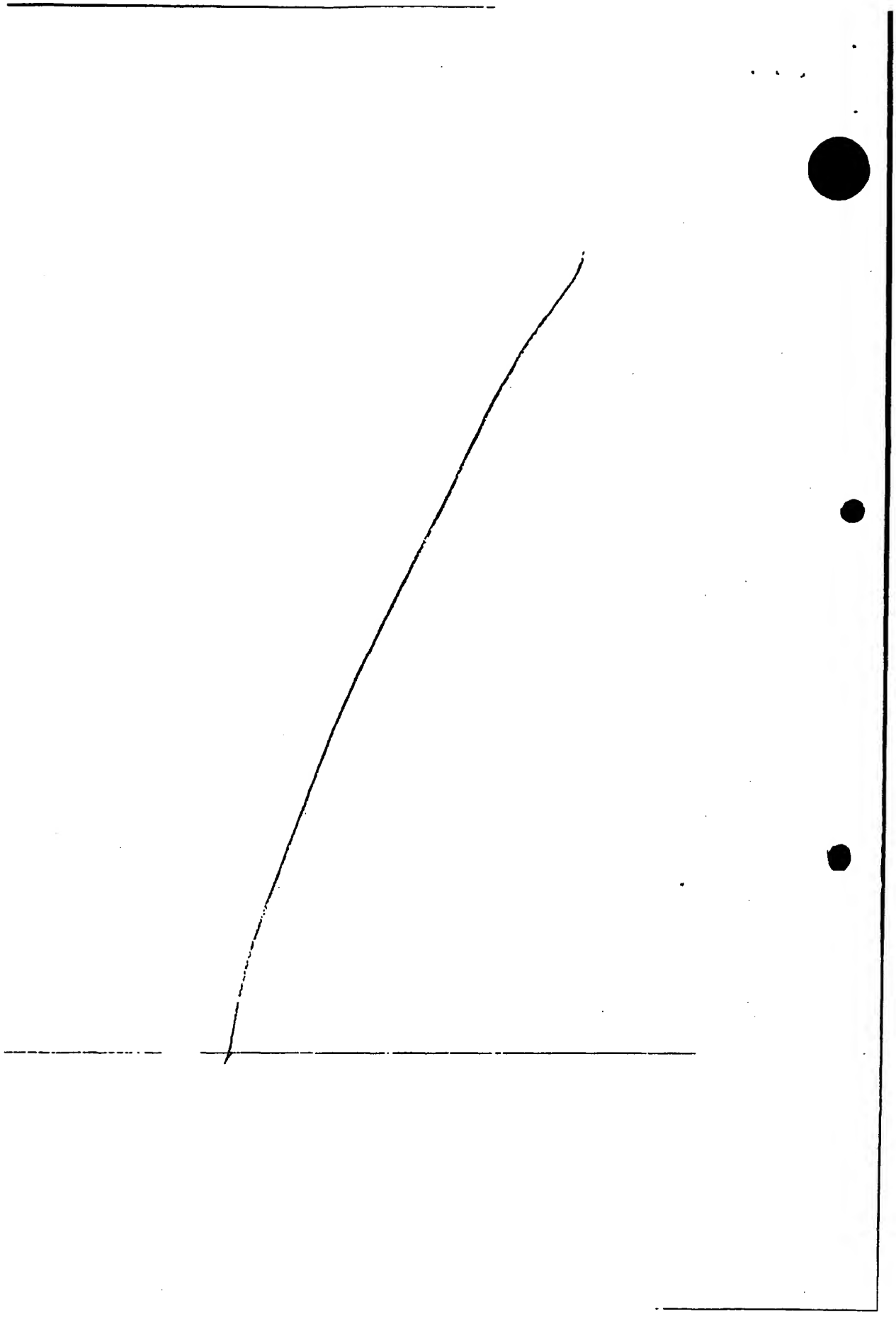

Residual activity = f(inhibitor concentration)
Residual activity, %
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
100
Inhibitor conc., ul

Fig. 12.



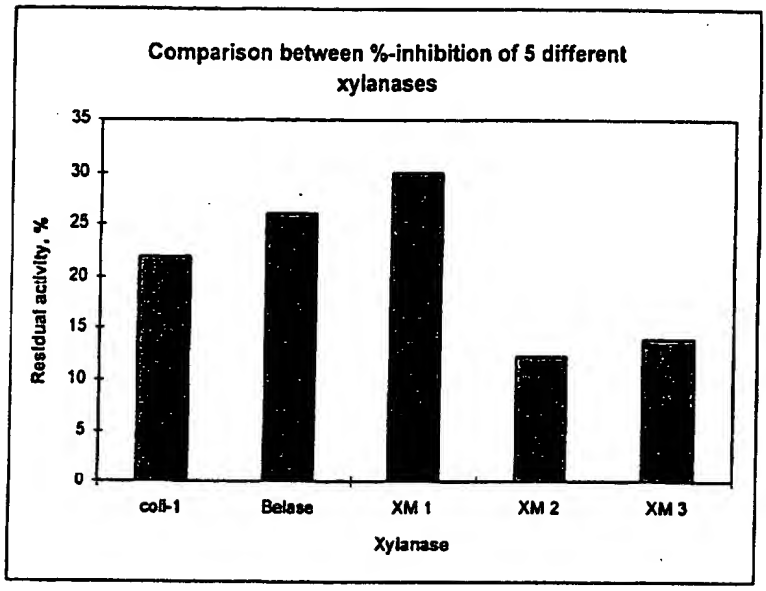
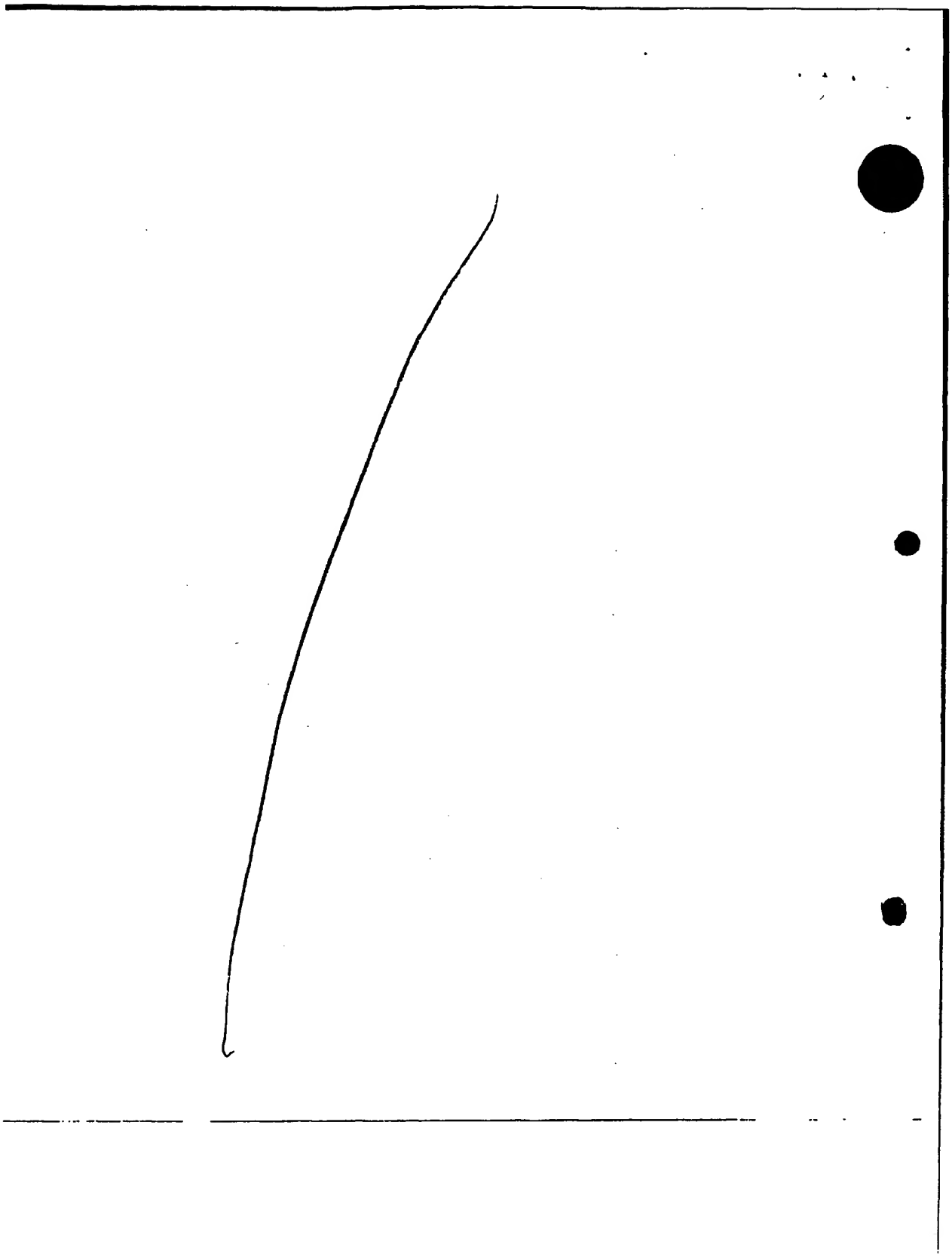

Comparison between %-inhibition of 5 different xylanases
Residual activity, %
35
30
25
20
15
10
5
0
coli-1
Belase
XM 1
XM 2
XM 3
Xylanase

Figure 13.



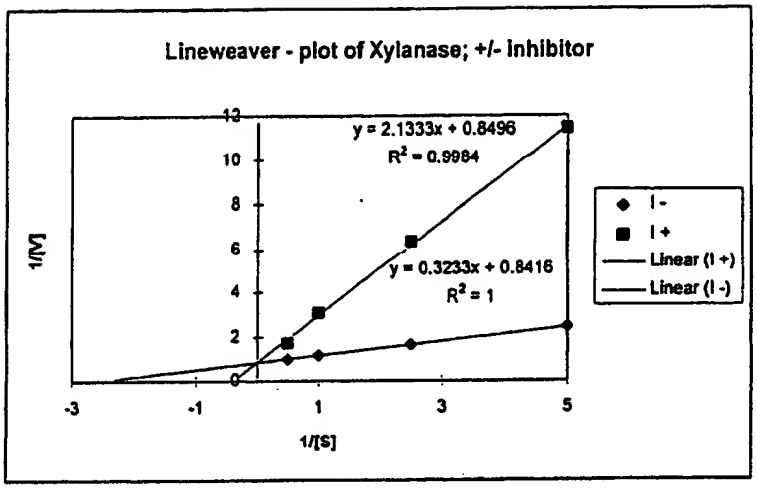
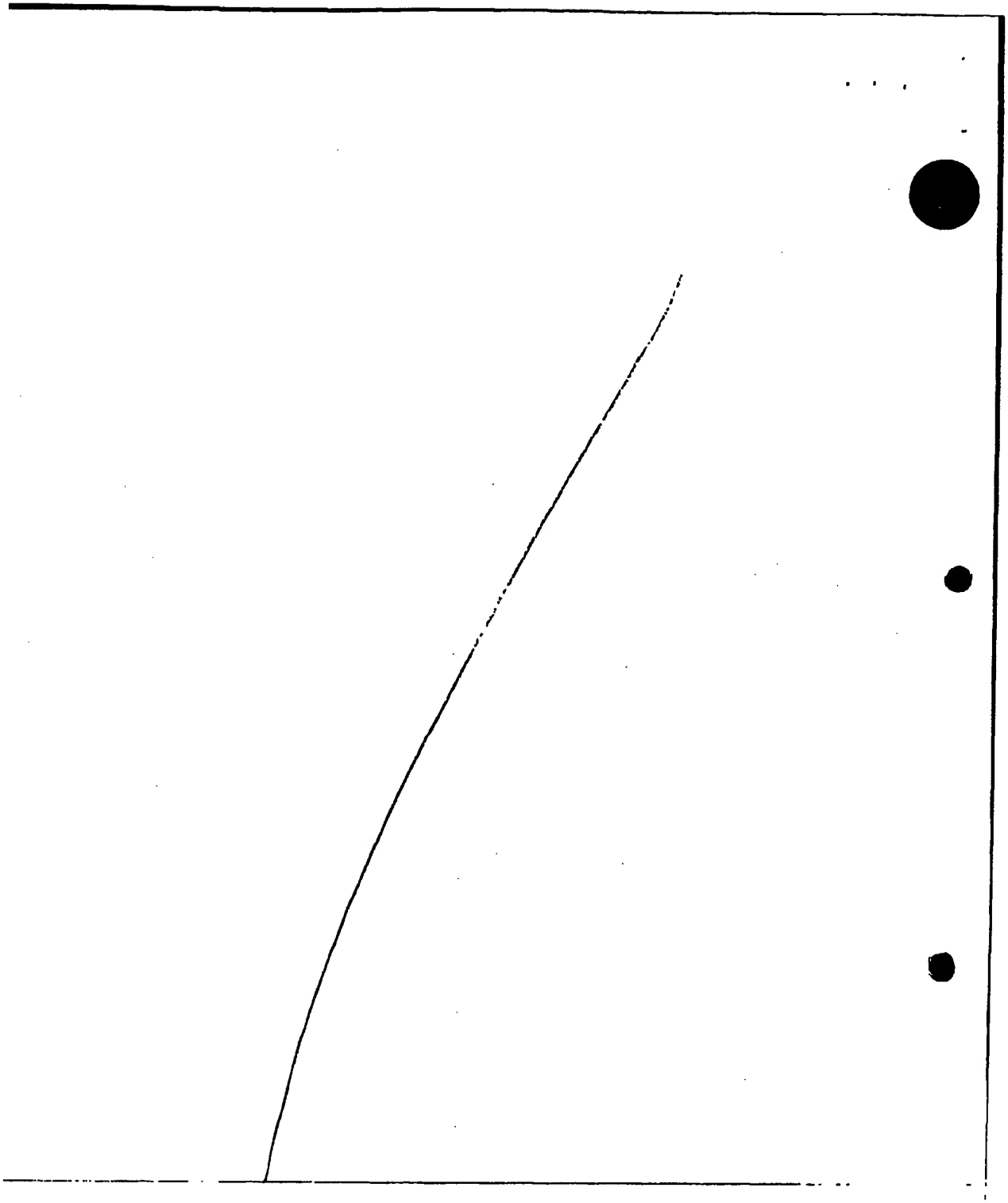

Lineweaver - plot of Xylanase; +/- Inhibitor
y = 2.1333x + 0.8496
R² = 0.9984
y = 0.3233x + 0.8416
R² = 1
I -
I +
Linear (I +)
Linear (I -)
1/[V]
1/[S]
12
10
8
6
4
2
0
-3
-1
1
3
5

Figure 14.



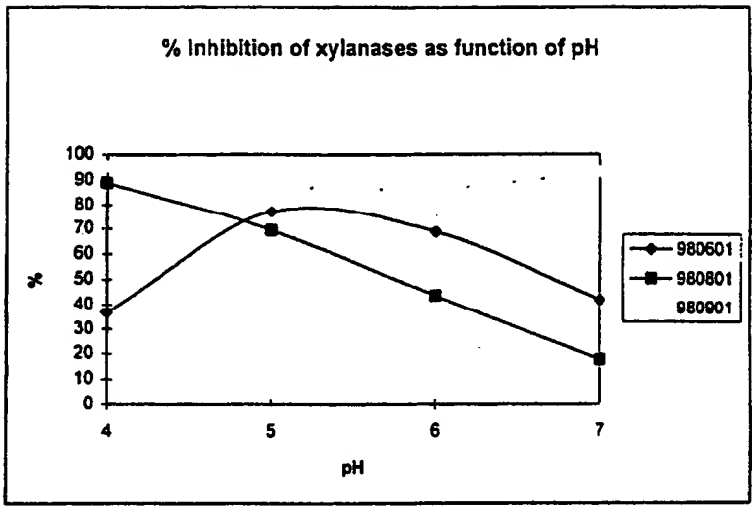
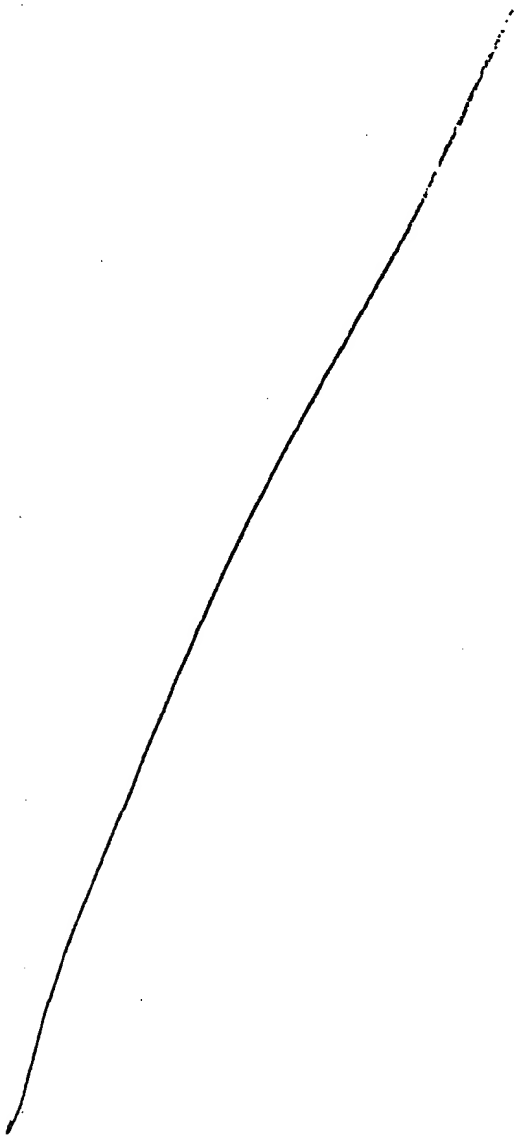

% Inhibition of xylanases as function of pH
100
90
80
70
60
50
40
30
20
10
0
%
4
5
6
7
pH
980601
980801
980901

Figure 15.



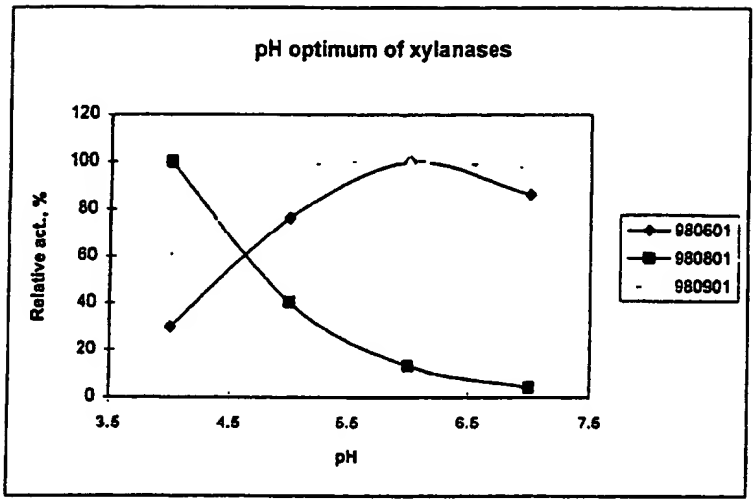
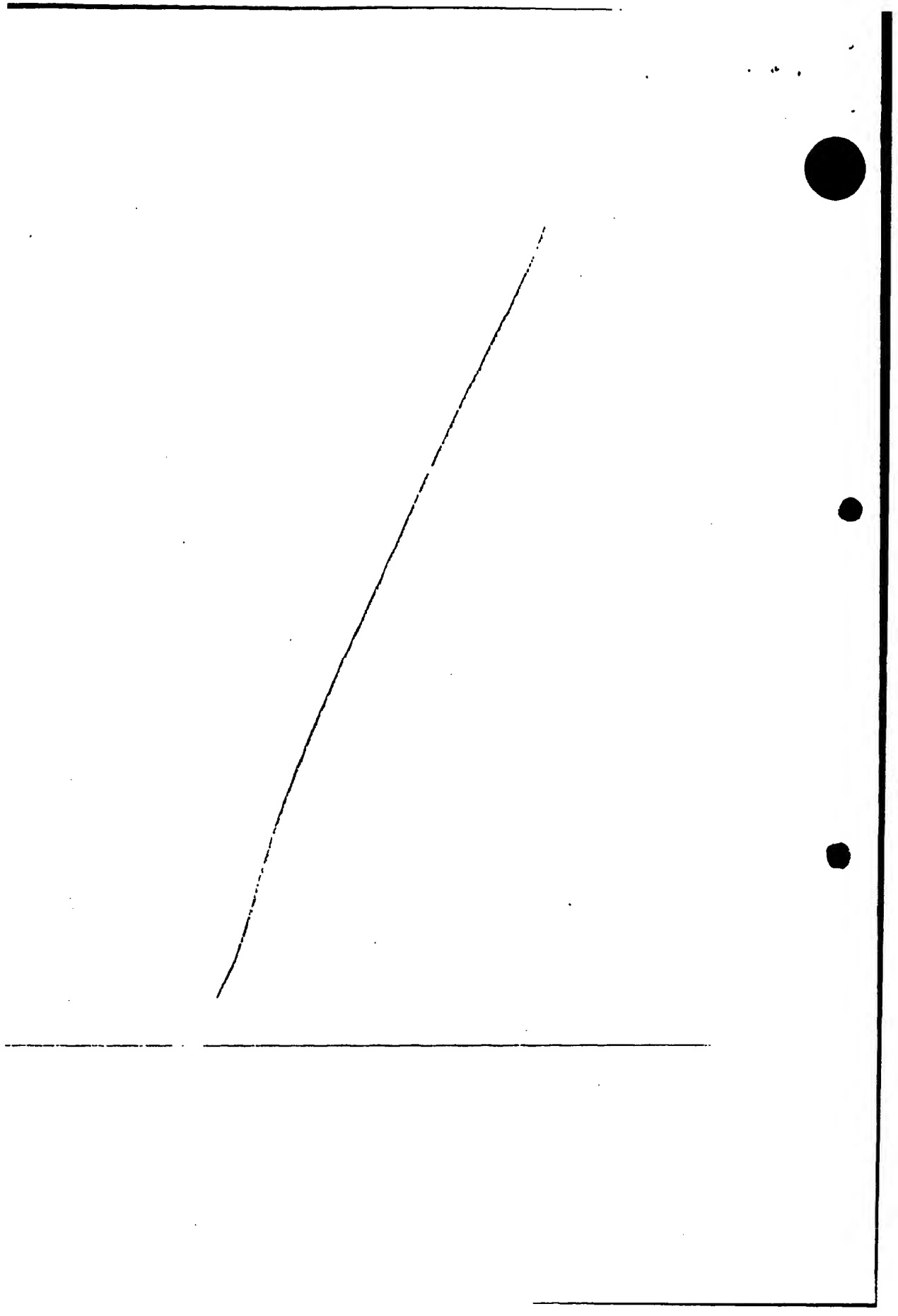

pH optimum of xylanases
Relative act., %
120
100
80
60
40
20
0
3.5
4.5
5.5
6.5
7.5
pH
980601
980801
980901

Figure 16.



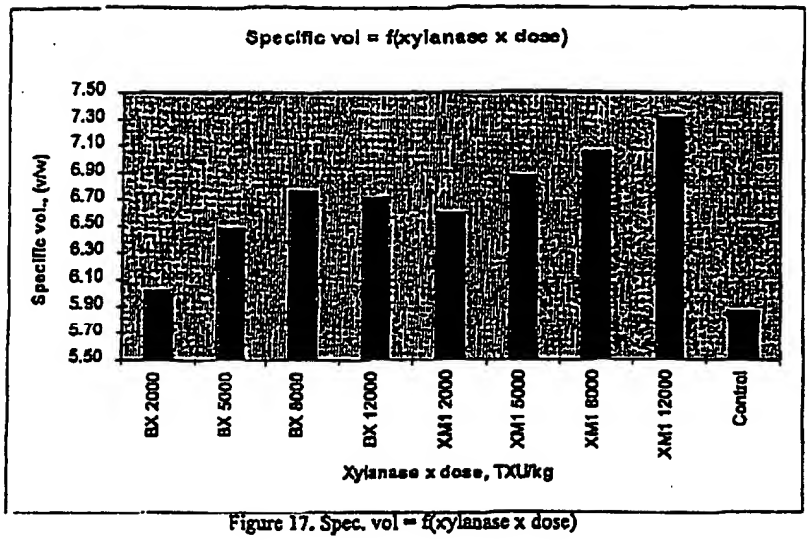
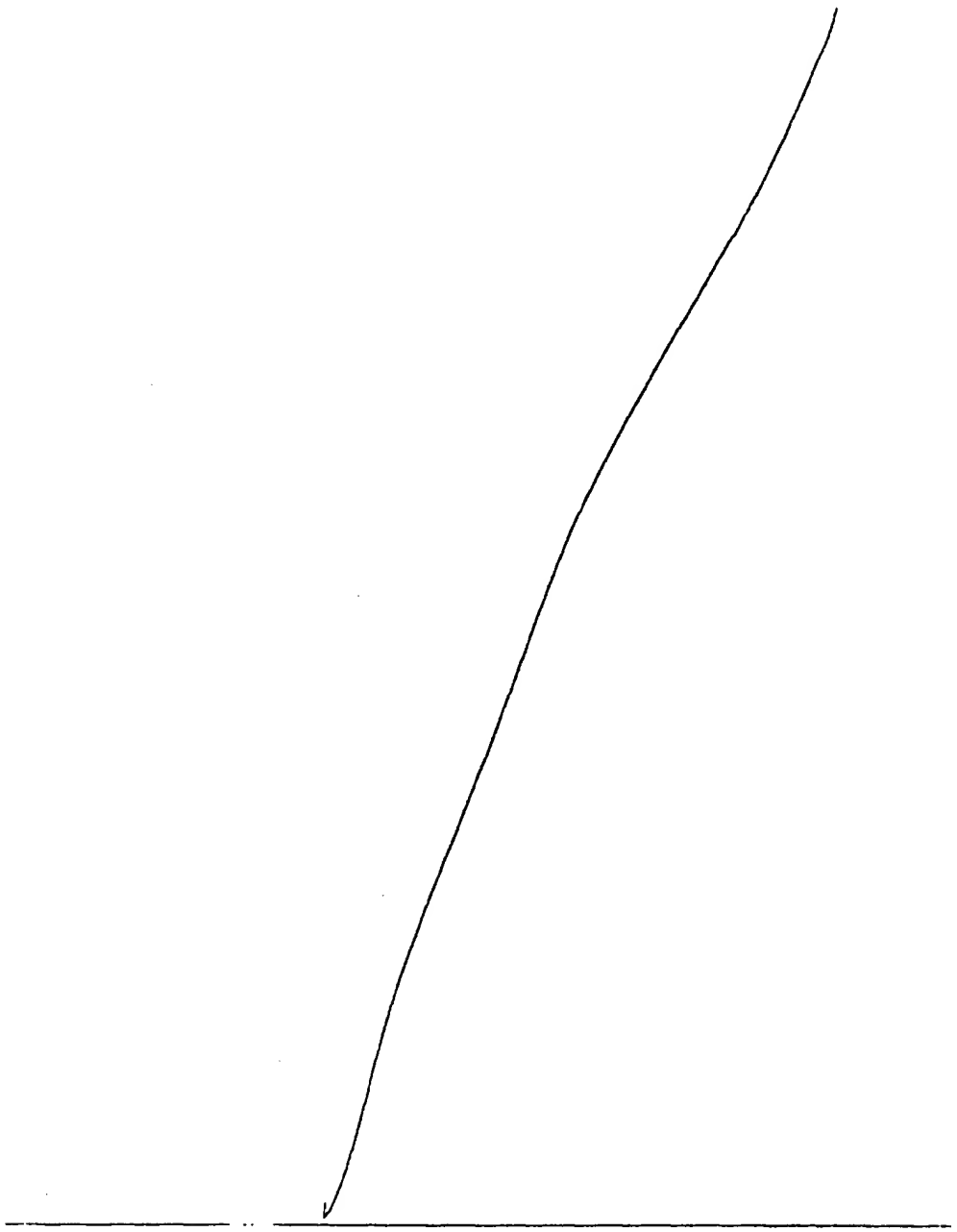


Figure 17. Spec. vol = f(xylanase x dose)



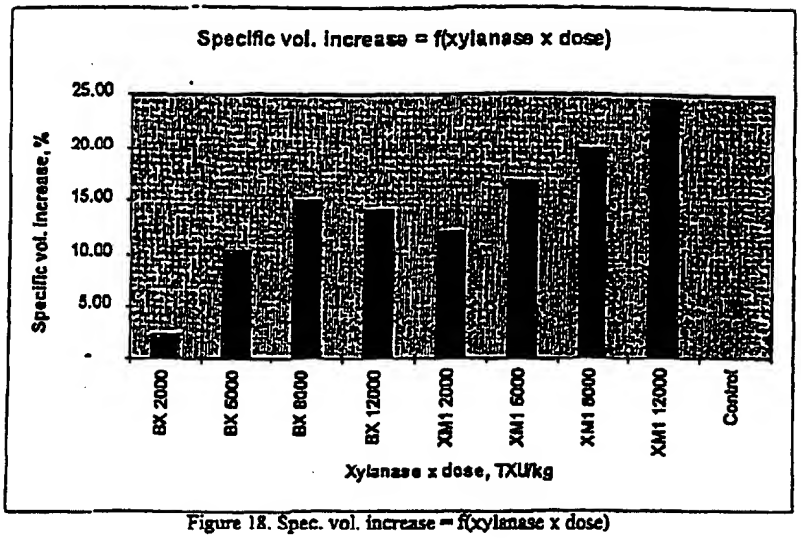
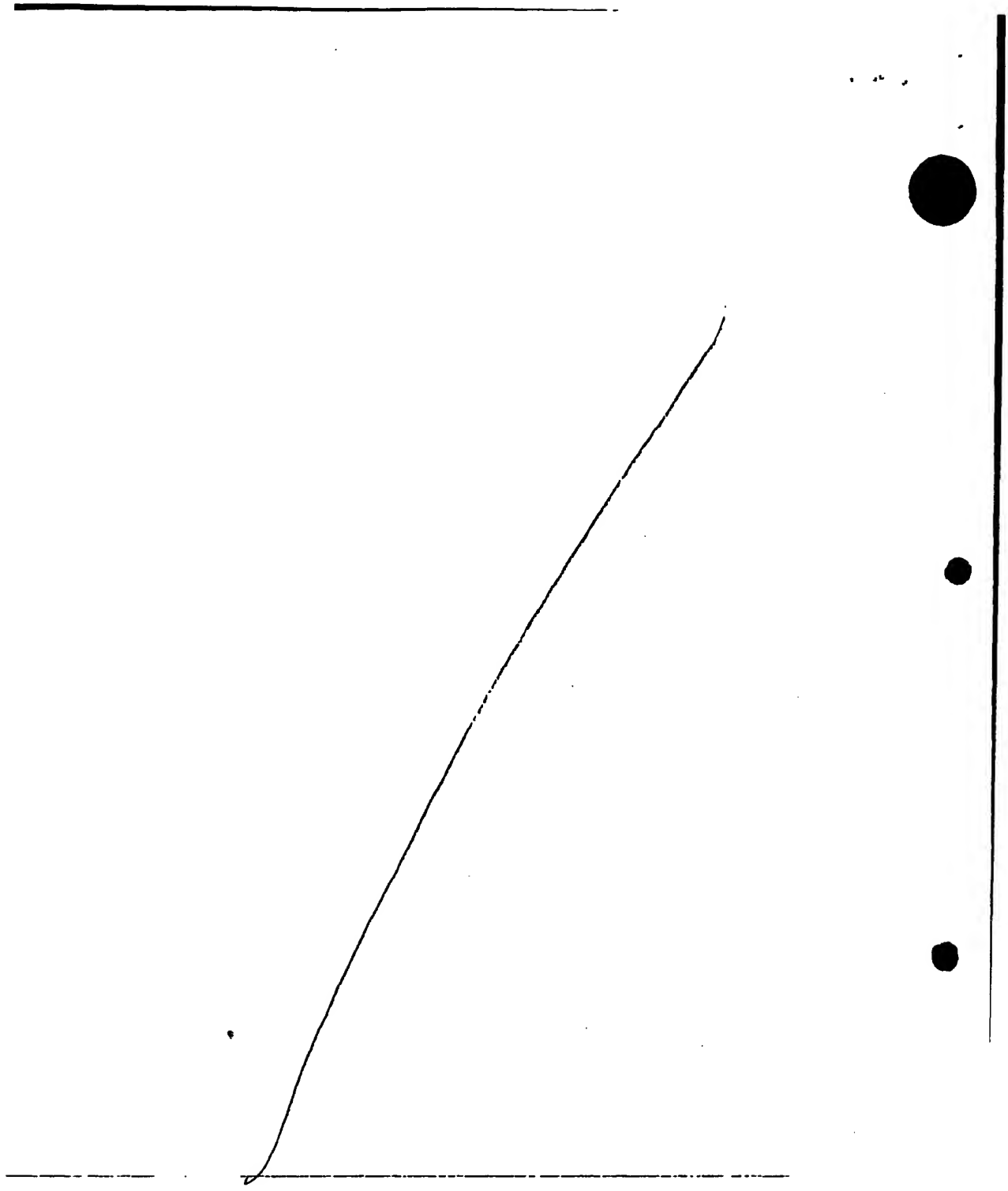


Figure 18. Spec. vol. increase = f(xylanase x dose)



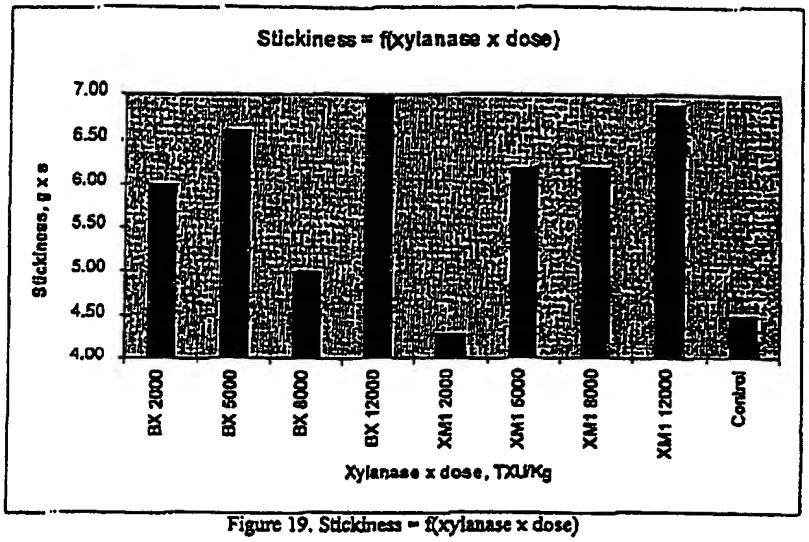
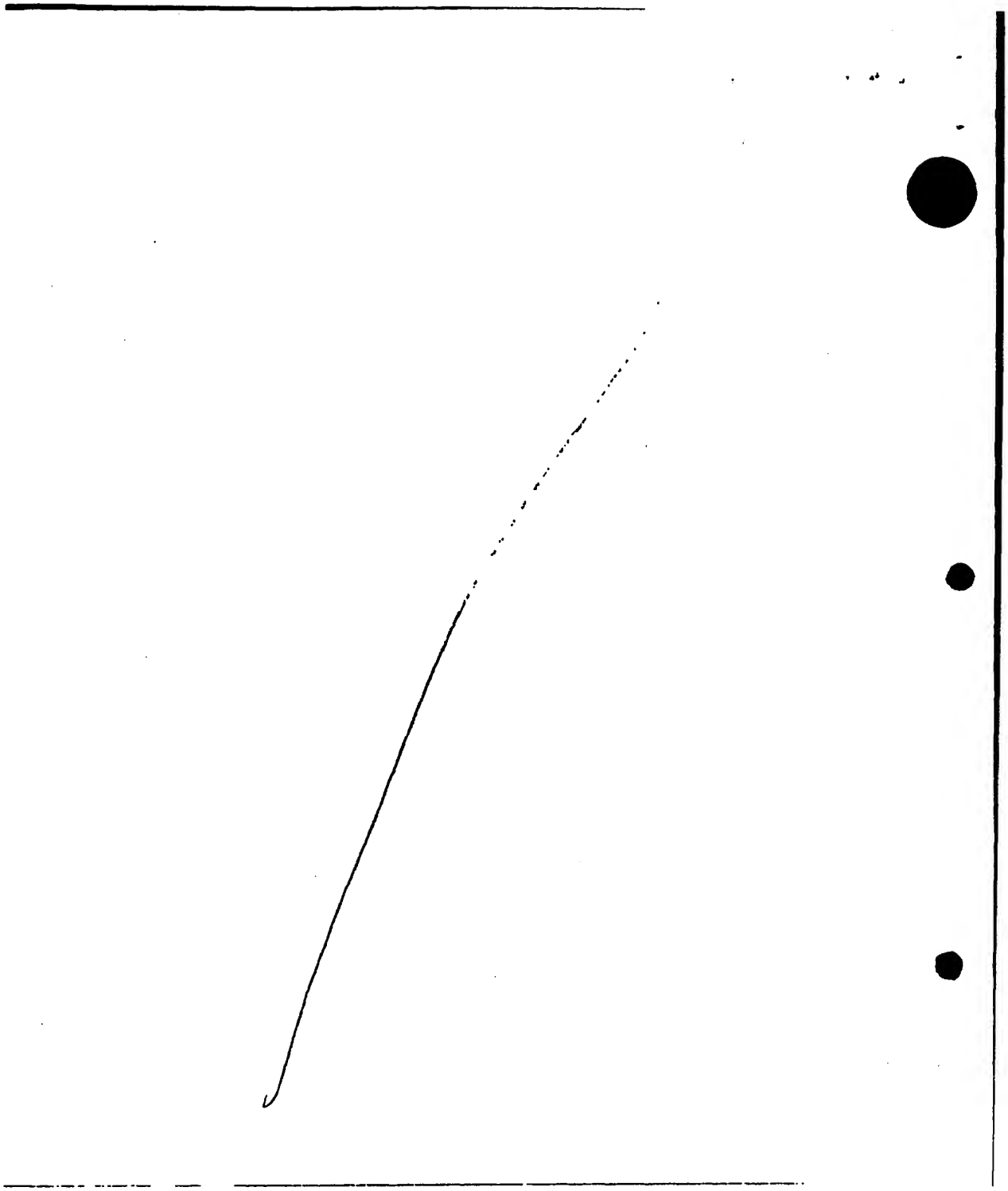


Figure 19. Stickiness = f(xylanase x dose)



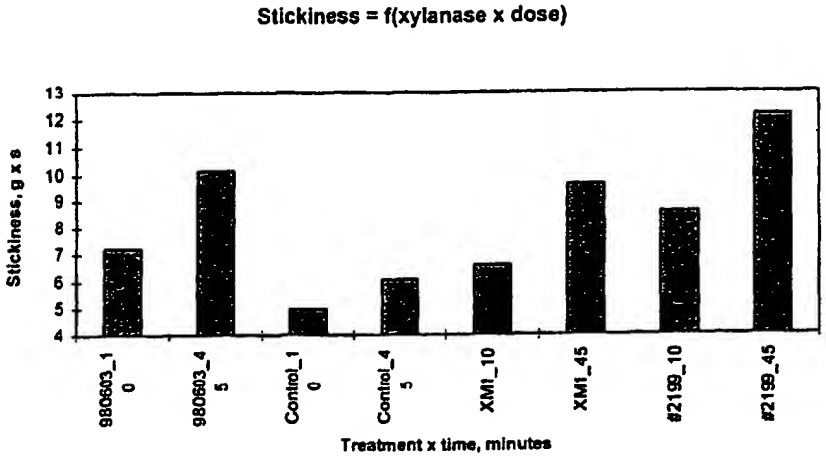
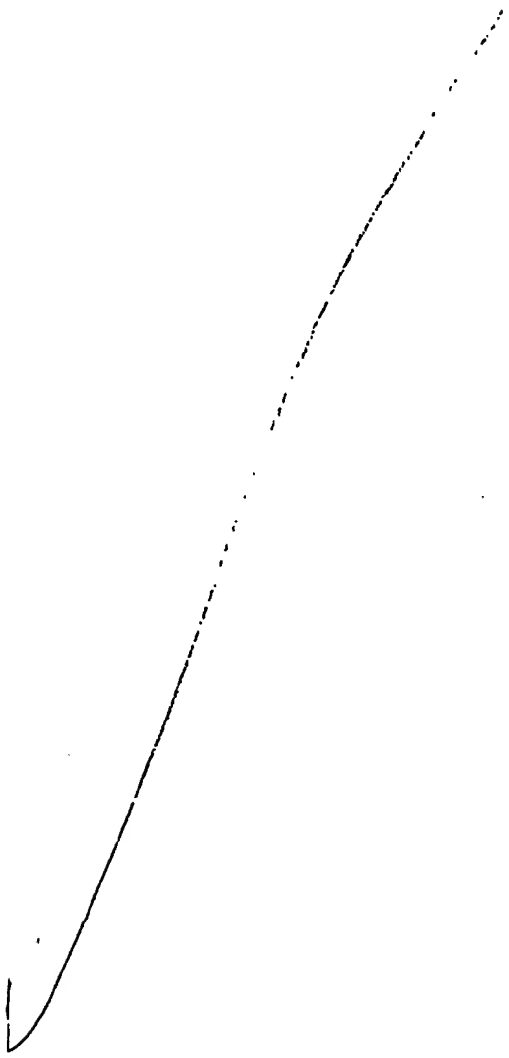

Stickiness = f(xylanase x dose)
Stickiness, g x s
13
12
11
10
9
8
7
6
5
4
980603_10
980603_45
Control_10
Control_45
XMI_10
XMI_45
#2199_10
#2199_45
Treatment x time, minutes

Figure 20.



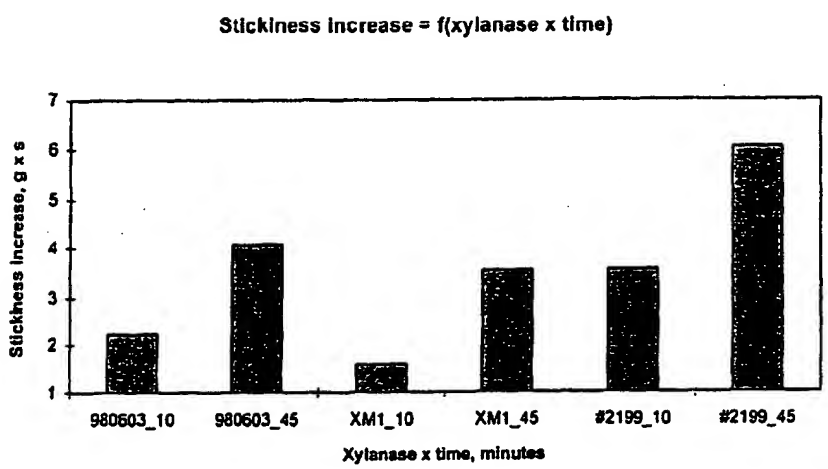
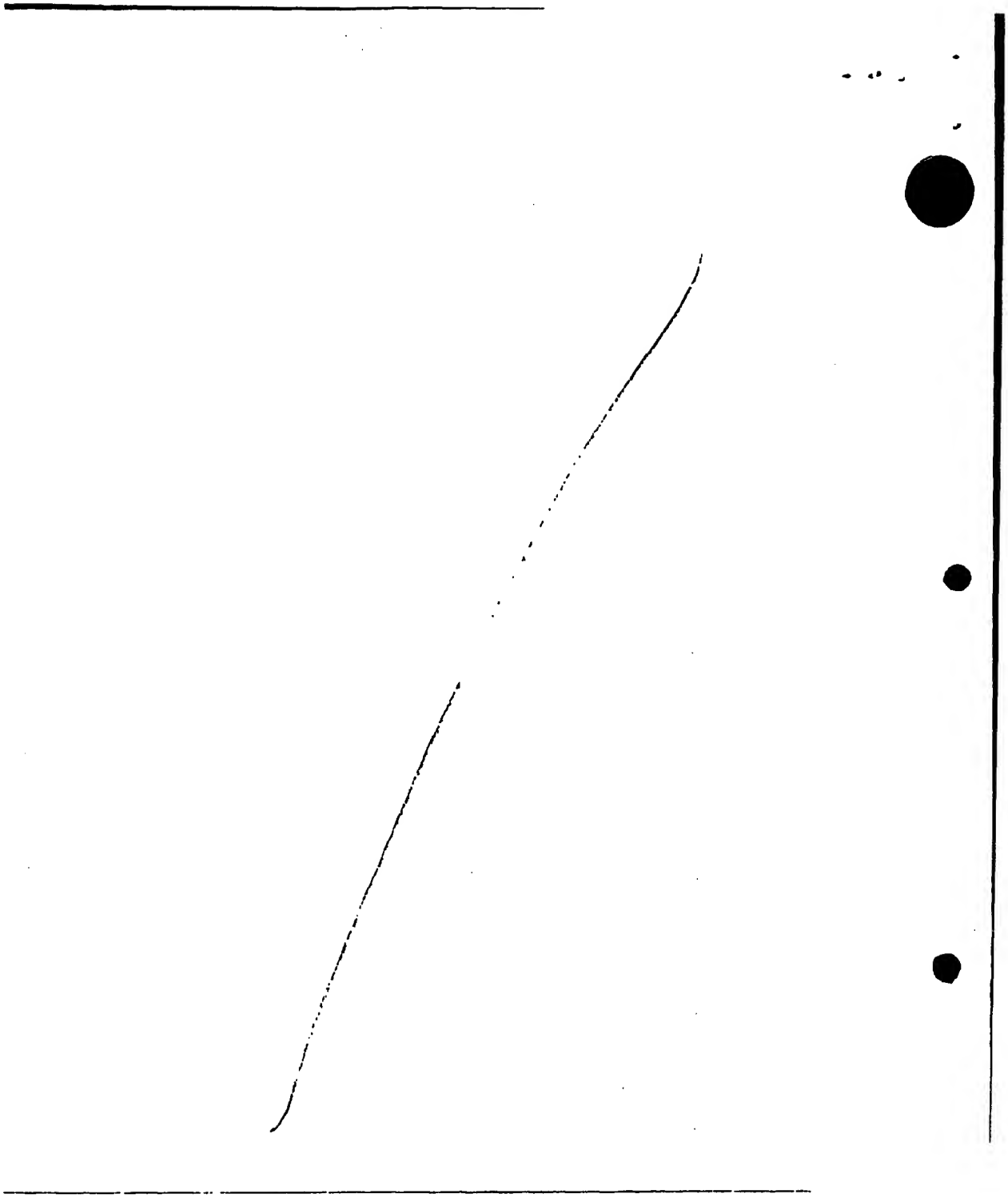

Stickiness increase = f(xylanase x time)
Stickiness increase, g x s
7
6
5
4
3
2
1
980603_10
980603_45
XM1_10
XM1_45
#2199_10
#2199_45
Xylanase x time, minutes

Figure 21.



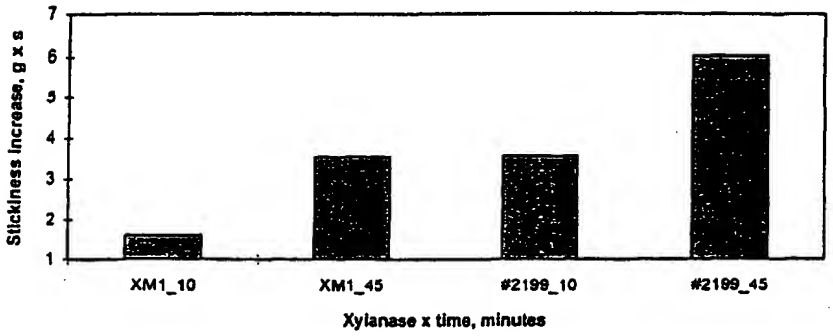
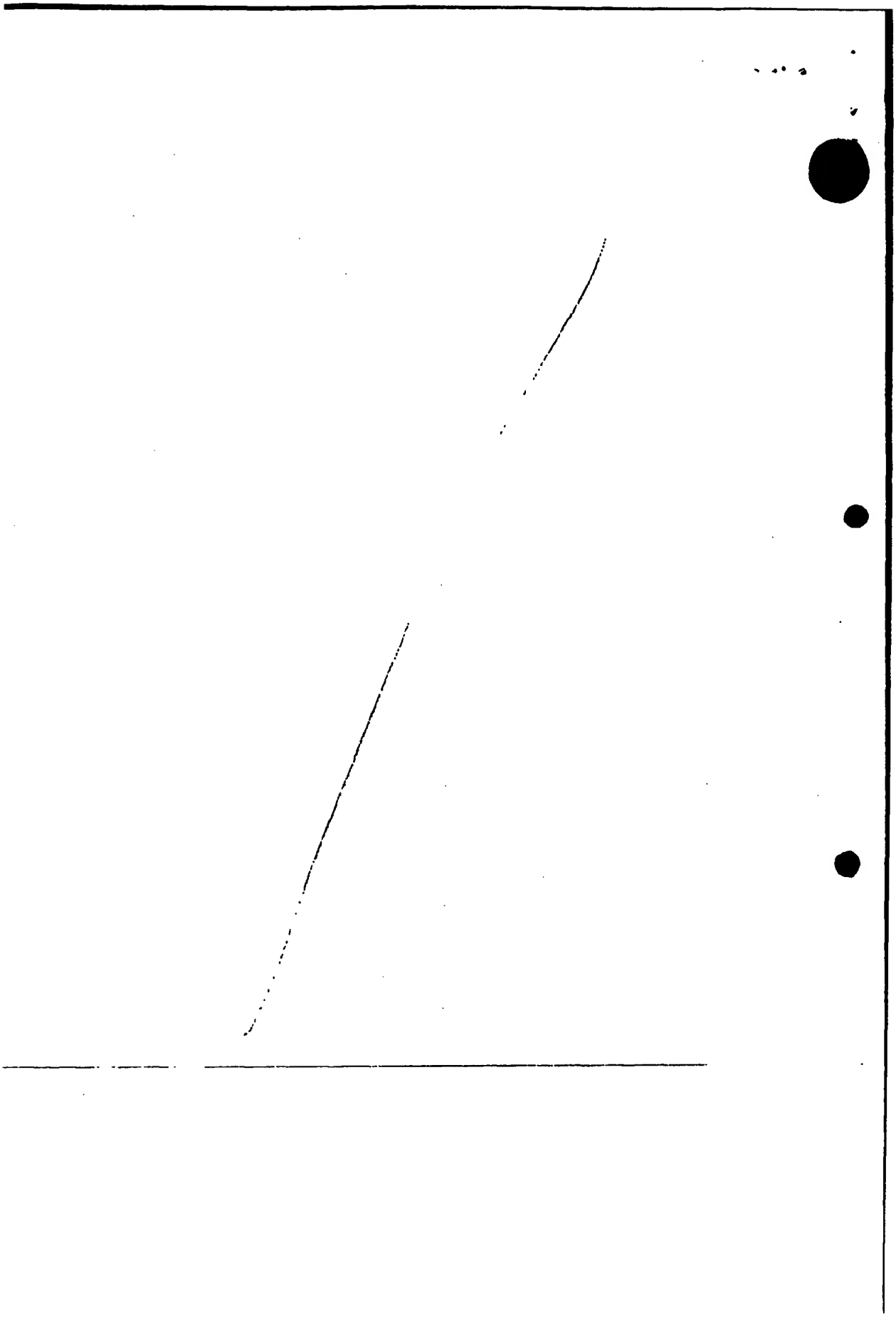

Stickiness increase = f(xylanase x time)
Stickiness increase, g x s
7
6
5
4
3
2
1
XM1_10
XM1_45
#2199_10
#2199_45
Xylanase x time, minutes

Figure 22.



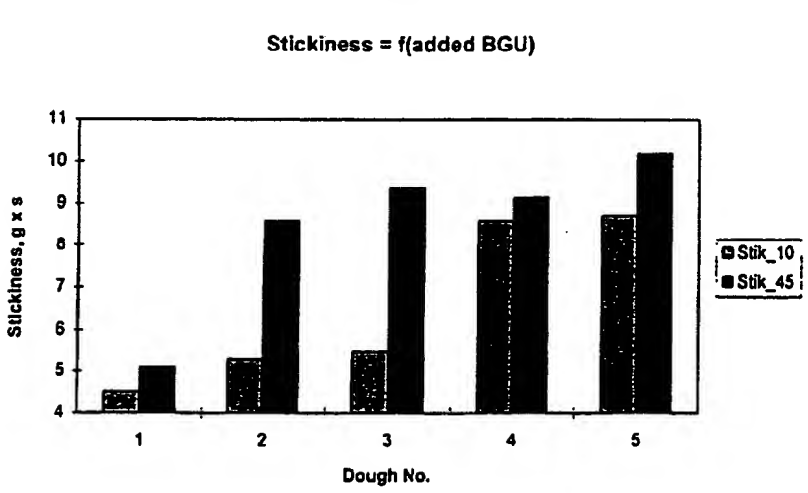

Stickiness = f(added BGU)
Stickiness, g x s
11
10
9
8
7
6
5
4
1
2
3
4
5
Dough No.
Stik_10
Stik_45

Figure 23.